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The Bacterial Catabolism of 2-Methylalanine Characteristics of the Enzyme 2-Methylalanine Decarboxylase.

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THE BACTERIAL CATABOLISM OF 2-METHYLALANINE; CHARACTERISTICS OF THE ENZYME 2-METHYLALANINE DECARBOXYLASE

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ABSTRACT

Various methyl-substituted amino acids, notably 2-methylalanine (α-aminoisobutyric acid), have been used in the study of amino acid transport into cells. These amino acids have been considered to be model substrates which escape detectable metabolic alteration. It has been presumed that such amino acids fail to be degraded because of the absence of a hydrogen atom on the α carbon and subsequent impairment of the commonly used metabolic pathways of amino acids (e.g., transamination, oxidative deamination, and certain α-β eliminations). The metabolic disposition of amino acids having a tertiary α carbon atom was, therefore, of interest.

A soil bacterium, identified as a member of the genus Pseudomonas, was found to oxidize 2-methylalanine to 65 per cent of the theoretical value by an adaptive enzyme system. Manometric experiments with whole cells indicated that acetone, but not isopropylamine, was an intermediate in the oxidation of 2-methylalanine.

Cellular extracts produced carbon dioxide from 2-methylalanine when pyruvic acid was added to the reaction mixture. Dialysis against a buffer containing 0.2 M KCl stimulated the 2-methylalanine decarboxylating system, provided pyridoxal phosphate and pyruvic acid were supplied. The optimum pH for the reaction was 7.8. A study of the stoichiometry of this pyridoxal phosphate-dependent decarboxylation

indicated that acetone and carbon dioxide were the principal products. The reaction was considered to be dependent upon the transfer of the amino group of 2-methylalanine to pyridoxal phosphate; pyridoxamine phosphate thus formed was considered to participate in the transamination of pyruvic acid to alanine.

The enzyme, 2-methylalanine decarboxylase, was not totally substrate specific. Isovaline, 2-methylserine, and 2-methylmethionine together with α-ketobutyric acid and α-ketovaleric acid were accepted as substrates. Both D- and L-isovaline were oxidized by whole cells.

The enzyme was inhibited by divalent cations, carbonyl agents, and the characteristic pyridoxal phosphate inhibitors, cycloserine and penicillamine. 2-Methylalanine decarboxylase was not inhibited by sulfhydryl agents.

Loss of enzyme activity occurred during dialysis against dilute buffer. This was attributed, in part at least, to the removal of the coenzyme, pyridoxal phosphate. Inactivated enzyme could be reactivated providing pyridoxal phosphate and KCl were added during the first three hours of dialysis. Longer periods of dialysis resulted in an enzyme preparation which could not be reactivated. A relatively specific requirement for the potassium ion was demonstrated; sodium and lithium ions were inhibitory. The potassium ion was also found to protect 2-methylalanine decarboxylase from heat inactivation. Addition of 2-methylalanine, pyridoxal phosphate, and potassium

ion protected the enzyme maximally. The kinetics of heat inactivation were biphasic, indicating the decay of more than one heat susceptible center.

The enzyme was purified 10-fold by protamine sulfate treatment, heat treatment, and ammonium sulfate precipitation. The sedimentation constant, as determined by density gradient centrifugation, was 8.5. The apparent Michaelis constants for the co-substrates, 2-methylalanine and pyruvic acid, were found to be 4.5×10^{-3} and 2.5×10^{-3} M, respectively. The Michaelis constant of pyridoxal phosphate was determined to be 3.0×10^{-5} M.

The enzymatic decarboxylation of 2-methylalanine was felt to be the enzymatic equivalent of a non-enzymatic model reaction reported in the literature. The reaction was considered to proceed via Schiff's base formation between 2-methylalanine and the coenzyme, decarboxylation, stabilization of the resulting substrate-coenzyme complex as the Schiff's base of the amine form of the coenzyme and acetone, followed by hydrolysis to yield acetone and the pyridoxamine form of the enzyme. Presumably the pyridoxal form of the enzyme was regenerated by the transamination of pyruvate to alanine. The reaction may be described as a decarboxylation dependent transamination.

INTRODUCTION

An investigation of the microbial attack on methyl-substituted amino acids, such as 2-methylalanine (α-aminoisobutyric acid), was initiated after three major considerations. These were: (1) the resistance of methyl-substituted amino acids to metabolic attack in many biological systems, (2) the possibility of studying the catabolism of a compound which was theoretically susceptible to metabolic attack by a number of different pathways, and (3) the possibility of studying the biochemistry of a compound for which a non-enzymatic reaction had been demonstrated.

The metabolic resistance ascribed to methyl-substituted amino acids (Christensen et al., 1952) has been attributed to the absence of the α hydrogen atom with subsequent impairment of pyridoxal phosphate enzyme systems. Methyl-substituted amino acids have been used largely as inhibitors of amino acid metabolizing enzymes (Umbreit, 1955) and as tracer metabolites in the study of amino acid transport (Christensen and Riggs, 1956).

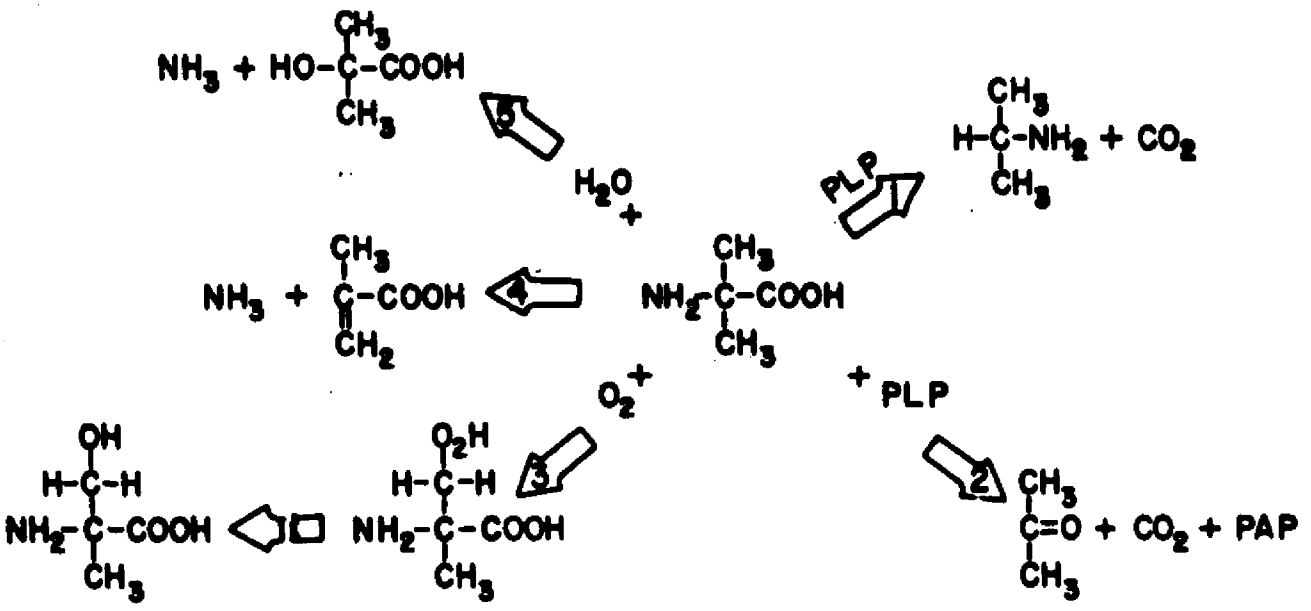
2-Methylalanine possesses two functional groups, a carboxyl and an amino group, plus two methyl groups. Although oxidative deamination or transamination would not be possible by the usual mechanisms, removal of the amino group could proceed via either hydrolytic or desaturative deamination. Decarboxylation could conceivably occur

resulting in the production of isopropylamine. A third possible metabolic pathway could proceed through the formation of an organic peroxide resulting from the incorporation of molecular oxygen into one of the methyl groups of 2-methylalanine. Metabolic pathways are reported in the literature by which any one of the first intermediates (methacrylic acid, 2-hydroxyisobutyric acid, isopropylamine, or 2-methylserine) of 2-methylalanine catabolism could be further metabolized.

An additional possibility concerning initial enzymatic attack on 2-methylalanine was suggested (Kalyankar and Snell, 1962) by the observation of a non-enzymatic, pyridoxal-catalyzed, decarboxylation dependent transamination. In this case acetone would be a first intermediate. The possible points of enzymatic attack on 2-methylalanine are summarized in Figure 1.

The purpose of this dissertation was (1) to isolate from nature an organism capable of utilizing 2-methylalanine as a sole source of carbon, (2) to determine the mechanism by which this compound was catabolized, and (3) to purify and characterize the pertinent enzyme or enzymes involved.

Figure 1. Possible points of metabolic attack on 2-methylalanine. (1) Decarboxylation to isopropylamine, (2) Decarboxylation dependent transamination to acetone and carbon dioxide, (3) Peroxidation or oxygenation eventually resulting in 2-methylserine, (4) Desaturative deamination to methacrylic acid, (5) Hydrolytic deamination to 2—hydroxyisobutyric acid. PLP and PAP refer to pyridoxal phosphate and pyridoxamine phosphate, respectively.


NH3 + HO-C(CH3)2-COOH
5
H2O +
PLP
H-C(CH3)-NH2 + CO2
NH3 + C(CH3)(=CH2)-COOH
4
NH2-C(CH3)2-COOH
O2 +
+ PLP
3
2
H-C(OH)-H
NH2-C-COOH
CH3
H-C(O2H)-H
NH2-C-COOH
CH3
C(=O)(CH3)2 + CO2 + PAP

REVIEW OF LITERATURE

Published reports have shown that amino acids, both dextro- and levo-rotary, may be attacked by biological systems in many ways. Deamination or transamination results in the removal or transfer of an amino group. Amino acid decarboxylases catalyze the removal of the α carboxyl group; however, exceptions to this occur in the decarboxylation of dicarboxylic amino acids. Although deamination, decarboxylation, and transamination are ubiquitous biochemical reactions, amino acid racemizations are peculiar to the bacteria. All of these general types of reactions must be considered when the bacterial catabolism of an amino acid is to be studied. It is significant that pyridoxal phosphate has been demonstrated to be the coenzyme in nearly all of these catabolic reactions and is also important in amino acid anabolism and the transport of amino acids across cellular membranes.

The degradative metabolism of amino acids most often involves the separation of the amino group from the amino acid. The resulting carbon skeleton may be converted to products in common with those of fat and carbohydrate metabolism, ultimately to be oxidized to completion or channeled into anabolic pathways. The general aspects of amino acid metabolism have been outlined in numerous symposia and texts (McElroy and Glass, 1955; Meister, 1957; Greenberg, 1961;

Sakami and Harrington, 1963). It is apropos, however, to briefly discuss the historical background of particular catabolic amino acid mechanisms.

Aside from the oxidative removal of the amino group, first definitively studied by Krebs (1935), processes of hydrolytic and desaturative deamination have been investigated. An example of the former is the hydrolytic deamination of L-aspartic acid to yield the hydroxy derivative, i. e., malic acid and ammonia as reported by Virtanen and Erkama (1938) in Pseudomonas fluorescens. Subsequent attempts by these same workers to verify such a hydrolytic deamination met with equivocal results (Erkama and Virtanen, 1951). Quastel and Woolf (1926) observed a desaturative deamination of L-aspartic acid in resting cells of Escherichia coli. The enzyme, aspartase, catalyzed the reversible reaction whereby fumaric acid and ammonia were produced. At the present time aspartase has been demonstrated only in microorganisms and plants. Other mechanisms of non-oxidative deamination have been observed. Metzler and Snell (1952a) showed that serine could be catabolized in Pseudomonas aeruginosa by enzymatic dehydration and subsequent deamination. The deamination of threonine, homoserine, cysteine, and homocysteine involves elimination of the elements of water or hydrogen sulfide from the amino acid. Such an elimination results in an α-β or a β-γ unsaturation. Rearrangement of the corresponding imino acid, followed by hydrolysis, would result in the production of an α-keto acid and ammonia.

Transamination, the transfer of an amino group from an amino acid to a keto acid without the intermediate participation of free ammonia, was first observed in 1934 (Herbst and Engel). They demonstrated such a reaction in boiling aqueous solutions. Enzymatic transamination was first reported by Braunstein and Kritsmann (1937) in pigeon breast muscle. Numerous studies have shown that the scope of enzymatic transamination is very broad. Transaminases specific for L-amino acids are found in all living systems; however, D-amino acid transminases have been demonstrated mostly in bacteria (Thorne, Gomay, and Housewright, 1955; Kuramitsu and Snoke, 1962). A recent report has shown that the serine transhydroxymethylase from rabbit liver can transaminate D-alanine (Schirch and Jenkins, 1964).

Amino acid decarboxylases attack amino acids yielding carbon dioxide and the corresponding amine. Amine oxidases have been demonstrated in bacteria (Satake, Ando, and Fujita, 1953; Martinez-Carrion and Jenkins, 1963) indicating that the production of amines is not a terminal reaction. Most bacterial decarboxylases are adaptive enzymes and have been shown to be formed in quantity only when the organisms had been grown in an acid medium (Gale, 1946). Seaman (1960) showed that decarboxylase production by Pseudomonas reptilivora was accompanied by alkaline conditions in the medium. The system studied by Ekladius, King, and Sutton (1957) differed from most bacterial decarboxylases in being optimally active near pH 7.0

and in not requiring acid conditions for its formation. Although there has been no completely satisfactory explanation of the function of amino acid decarboxylases in the metabolic operation of bacterial cells, it has been suggested (Gale, 1946) that the products of the reaction serve to neutralize a strongly acid medium and to supply a source of carbon dioxide for fixation reactions under conditions of limited carbon dioxide retention.

The mutual oxidation-reduction of pairs of amino acids (Stickland reaction) resulting in their deamination occurs in Clostridium sporogenes (Stickland, 1934). Many clostridia growing on protein hydrolyzates or amino acid mixtures appear to obtain most of their energy by such a catabolic mechanism with certain amino acids acting as hydrogen donors and others as hydrogen acceptors.

Enzymes which racemize amino acids have been demonstrated in microorganisms. Wood and Gunsalus (1951) partially purified an enzyme from Streptococcus faecalis which racemized D- and L-alanine. These workers first proposed the term, racemase, for this type of enzyme. The presence of alanine racemase appears to be widespread in microorganisms. It has been shown that alanine racemase coupled with specific D-amino acid transaminases can account for the synthesis of those D-amino acids present in the cell wall material (mucopeptide) of bacteria. This subject has been reviewed by Salton (1964).

The importance of the coenzyme, pyridoxal phosphate, is well documented for enzymatic reactions involving amino acid transaminations,

deaminations (dehydrases and desulfhydrases), decarboxylations, and racemizations. Schlenk and Snell (1945), Green, Leloir, and Nocito (1945), and Lichstein, Umbreit, and Gunsalus (1945) have reported experiments which led directly to the recognition of the essential role of pyridoxal phosphate in enzymatic transamination. Umbarger and Brown (1956) have observed L- and D-threonine dehydrases in E. coli which were stimulated by pyridoxal phosphate. Likewise the preparation of D-serine dehydrase from E. coli has been shown to require pyridoxal phosphate (Metzler and Snell, 1952a). The properties of amino acid decarboxylases have been reviewed (Gale, 1946; Snell, 1953) and pyridoxal phosphate has been reported to be the coenzyme. In the first published report on alanine racemase (Wood and Gunsalus, 1951), pyridoxal phosphate was shown to be the required coenzyme.

The mechanism of pyridoxal-catalyzed reactions has been carefully studied (Metzler and Snell, 1952b; Metzler, Ikawa, and Snell, 1954). The similarity between reactions catalyzed by enzymatic systems and those catalyzed non-enzymatically by heated solutions of pyridoxal and metal salts has been exploited. The structural features of the pyridoxal molecule required for catalysis of these non-enzymatic reactions are the formyl group, the phenolic group, and the heterocyclic-ring nitrogen arranged in the 4-, 3- and 1-positions, respectively. The formyl group appears to function in formation of a Schiff's base with the amino acid. The pyridoxal-amino acid complex is felt to be stabilized

by chelation with the catalytic metal ion (in non-enzymatic reactions) or the apoenzyme (in enzymatic reactions) (Snell and Jenkins, 1959), via the nitrogen of the resulting azomethine linkage, the phenolic group, and probably the carboxyl group of the amino acid. The resulting planar system of conjugated double bonds provides a mechanism for the displacement of an electron pair from any of the bonds of the α carbon of the amino acid toward the strongly electrophilic heterocyclic nitrogen. Various structurally and electronically equivalent compounds such as 2-formyl-3-hydroxypyridine have been shown to be active (Metzler et al., 1954). Thus, transamination, the elimination of an α hydrogen, together with a β substituent, decarboxylation and/or racemization of the amino acid may take place. The reverse of the α-β elimination has also been demonstrated, as in the enzymatic catalysis of tryptophan formation from serine and indole (Umbreit, Wood, and Gunsalus, 1946). Recently a most interesting observation by Cennamo (1964) has indicated that metal ion independent transamination may occur between pyridoxal and amino acid esters.

The general reaction mechanism of pyridoxal-catalyzed reactions, which may be considered to be analogous to the enzymatic mechanisms, have been summarized by Metzler et al. (1954). Release of a proton, the carboxyl group or the amino acid side chain from the pyridoxal-amino acid complex has been considered in detail. It is significant that loss of the α hydrogen atom is essential in reactions leading to

the racemization, transamination or α-β elimination of an amino acid. This is not the case if the release of the amino acid side chain, as in the cleavage of threonine to glycine and acetaldehyde (Metzler, Longenecker, and Snell, 1953), or the carboxyl group, is to be affected. The decarboxylation of an amino acid was not prevented by replacing the α hydrogen atom with an alkyl group (Metzler et al., 1954). The retention of the α hydrogen during enzymatic decarboxylation of lysine, tyrosine and glutamic acid in D_2O (Mandeles, Koppelman, and Hanke, 1954) is significant in this regard. A concise mechanistic review of pyridoxal phosphate enzymatic reactions has been presented by Kosower (1962) and a recent symposium (Snell et al., 1963) has reviewed chemical and biological aspects of pyridoxal catalysis.

2-Methyl amino acids are amino acid analogs. These compounds possess a methyl group in place of the hydrogen atom on the α(or 2) carbon atom of the amino acid. The most common examples of such compounds are 2-methylalanine (2-methyl-2-aminopropionic acid or α-aminoisobutyric acid) and isovaline (2-methyl-2-aminobutyric acid). den Dooren de Jong (1926) reported that these amino acids appeared to be degraded by bacteria. He showed that Aerobacter aerogenes, Bacterium herbicola and Mycobacterium phlei could grow in a medium containing 1 per cent glucose, 1 per cent 2-methylalanine, 0.1 per cent K_2HPO_4, and 1 per cent $CaCO_3$ in tap water. Isovaline likewise served as an apparent combined nitrogen source for Bacillus polymyxa,

Aerobacter aerogenes and Bacterium herbicola under similar conditions. 2-Methylalanine did not support growth of any of the above organisms when used as a sole source of carbon and nitrogen.

The metabolic fate of 2-methylalanine and isovaline in animal tissues was studied by Leighty and Corley in 1937. They reported that these amino acids were for the most part excreted in the urine after subcutaneous administration into dogs. Christensen, Aspen, and Rice (1956) reported that 2-methylalanine, 2-hydroxymethylserine, and 2-methyl-DL-serine were accumulated by the liver of the rat after intraperitoneal administration. Their urinary excretion was approximately 80 per cent in 15 to 30 hours. 2-Methylalanine was excreted to a minor extent in the conjugated form, in part as the acetyl derivative. This and earlier observations (Leighty and Corley, 1937) of the nonmetabolizability of 2-methylalanine made at high dose levels were confirmed by tracer studies using 2-methylalanine-1-C^{14} (Noall et al., 1957). Such studies also indicated that 2-methylalanine-1-C^{14} was not incorporated into protein in a mammalian protein synthesizing system. Christensen and Jones (1961) extended the use of labeled methyl-substituted amino acids. They reported that $C^{14}O_2$, detected by liquid scintillation counting, permitted measurement of a very limited metabolic breakdown of 2-methylalanine, 1-aminocyclopentane-1-carboxylic acid, and 1-aminocyclohexane-1-carboxylic acid following intraperitoneal injection into mice. These authors did not attribute this breakdown to

the animal organism since the contents of the lower intestine were able to release $C^{14}O_2$ from 2-methylalanine. A bacterial cell extract which released $C^{14}O_2$ and ammonia from 2-methylalanine-1-C^{14} was reported but no definitive study was made (Christensen and Jones, 1961).

Since methyl amino acids, such as 2-methylalanine, appear to be resistant to metabolic attack, at least in mammalian systems, they have often been employed as metabolic tracers to follow amino acid transport and concentration. Christensen et al. (1952) followed the incorporation of 2-methylalanine and isovaline into Ehrlich mouse ascites carcinoma cells, and Christensen and Riggs (1956) reported that these amino acids were concentrated to an even greater extent than their naturally occurring analogs by the same cells. Representative studies also include the work of Riggs and Walker (1958) in which diminished uptake of 2-methylalanine-1-C^{14} was observed by the tissues of rats deficient in vitamin B_6. General endocrine control of amino acid transfer (Noall et al., 1957) and the action of interstitial cell stimulating hormone (Hall and Eik-Nes, 1962) have been studied in animal tissues with 2-methylalanine-1-C^{14}. 2-Methylalanine has also been employed in the study of amino acid transport in bacteria (Drapeau and MacLeod, 1963; Marquis and Gerhardt, 1964).

Results indicating the chemical stability of 2-methylalanine were published by Leighty and Corley (1937). They reported only 66 per cent deamination of 2-methylalanine after 22 minutes contact with

nitrous acid. Additionally, Christensen et al. (1952) showed that methyl amino acids were degraded so slowly by ninhydrin that they could be determined by the carbon dioxide evolved after other amino acids had been completely degraded by ninhydrin.

Methyl-substituted amino acids have been found to serve as inhibitors in various areas of amino acid metabolism. This subject was reviewed by Clark (1963). 2-Methylglutamic acid has been found to inhibit transamination reactions (Braunstein, Azarkh, and Mogilevskaya, 1956) as well as decarboxylations (Roberts, 1953). Detailed study of the action of 2-methylglutamic acid on the glutamic decarboxylases of Lactobacillus arabinosus revealed its action as a competitive inhibitor (Roberts, 1953). Two aromatic methyl amino acids, 2-methyl-3,4-dihydroxyphenylalanine and 2-methyl-3-hydroxyphenylalanine, added at low concentrations, accelerated, and at high concentrations, inhibited pig kidney dihydroxyphenylalanine decarboxylase (Sourkes, 1954). 2-Methylmethionine has been shown to block D-amino acid oxidase action on phenylalanine (Pfister et al., 1955).

Although a resistance to general metabolic attack is a principal feature of methyl-substituted amino acids, a number of studies have been carried out to study their degradation. The earliest report of such catabolic activity was that of Ehrlich (1908). He reported the yeast fermentation of DL-isovaline and the isolation of the levorotary isomer from the spent culture medium. Subsequently den Dooren de Jong (1926)

indicated that a number of bacteria were able to employ either 2-methylalanine or isovaline as a source of combined nitrogen when grown in a medium containing 1 per cent glucose. The amino acids did not support growth of the test organisms in unsupplemented media.

The metabolic lability of 2-methylalanine was indicated by Christensen and Jones (1961) when they reported that, "numerous coliform organisms are able to degrade α-aminoisobutyric acid.....in the lower intestine" of the mouse. They further stated that a bacterial cell extract released $C^{14}O_2$ and ammonia from α-aminoisobutyric acid-1-C^{14}. A personal communication with Dr. Christensen (1964) revealed that no additional work concerning this phenomenon had been published.

Few definite studies on the metabolic fate of 2-methyl amino acids have been made. Weissbach, Lovenberg, and Undenfriend (1960) demonstrated that a mammalian amino acid decarboxylase acted on 2-methyltryptophan, 2-methyl-5-hydroxytryptophan, and 2-methyldihydroxyphenylalanine. Similarly Carlsson and Lindqvist (1962) reported the in vivo decarboxylation of 2-methyldihydroxyphenylalanine and 2-methylmetatyrosine in mice and rabbits after both intraperitoneal and intravenous injection. Hayaishi et al. (1961) reported that 2-methylalanine was transaminated at 4 per cent the rate of β-alanine by a α-alanine-β-alanine transaminase purified 80- to 150-fold from P. fluorescens.

Wilson and Snell (1962a, b) reported that 2-methylserine and 2-hydroxymethylserine were metabolized to alanine and serine, respectively,

by a soil pseudomonad. In the presence of added tetrahydrofolate crude extracts of this organism converted 2-methylserine to 5,10-methylenetetrahydrofolate and alanine. From such extracts a pyridoxal phosphate dependent enzyme, 2-methylserine hydroxymethyltransferase, was purified 25-fold. The work of Wilson and Snell (1962a,b) represents the enzymatic equivalent of the non-enzymatic pyridoxal-catalyzed cleavage of 2-methylserine studied by Longenecker, Ikawa, and Snell (1954).

Kalyankar and Snell (1962), employing a non-enzymatic system, showed that 2-methylalanine underwent two closely related but independent reactions when heated with pyridoxal in an aqueous solution in the absence of metal ions.

(1) $(CH_3)_2\text{-}C(NH_2)\text{-}COOH \xrightarrow{\text{pyridoxal}} (CH_3)_2\text{-}CH\text{-}NH_2 + CO_2$

(2) $(CH_3)_2\text{-}C(NH_2)\text{-}COOH$ + pyridoxal $\longrightarrow$ $(CH_3)_2\text{-}C{=}O + CO_2$ + pyridoxamine

Reaction 1 is analogous to the decarboxylation of amino acids by pyridoxal phosphate enzymes; reaction 2 was described as a decarboxylation dependent transamination.

MATERIALS AND METHODS

Bacteriological

Isolation of the experimental organism. The technique of enrichment culturing was employed in this study in order to isolate an organism which was capable of utilizing 2-methylalanine as a sole source of carbon. One gram samples of soil obtained from different areas on the campus of Louisiana State University were used as a source of inoculum. The enrichment medium contained 0.2 per cent 2-methylalanine in the salt solution of Stanier (1947). The pH of the medium was 7.0. Each soil sample was inoculated into a 250 ml Erlenmeyer flask containing 100 ml of medium. After three to five days of incubation at room temperature (approximately 25 C), growth was observed and 0.5 to 1.0 ml of culture was transferred to fresh medium and again incubated. A loopful of material from the fourth transfer was streaked for colony isolation on a solid medium dispensed in petri dishes. The solid medium was identical to the enrichment medium except that it contained 1.5 per cent agar. Samples of colonial growth were restreaked to insure purity of the isolates. Isolates were examined by routine bacteriological methods with respect to colonial and cellular morphology, and staining.

2-Methylalanine metabolizing isolates were carried in screw cap test tubes in media of the following composition:

2-Methylalanine Medium

Compound	Per cent
2-Methylalanine	0.2
Sodium acetate	0.005
Yeast extract (Difco)	0.005
K_2HPO_4	0.1
NH_4NO_3	0.1
$MgSO_4$	0.05
Agar (Difco)	1.5

Solvent: Tap water pH 7.2

Cultures were transferred every two weeks on the average and periodically reexamined microscopically. Incubation of cultures and of all culturing to be subsequently mentioned was carried out at 30 C except for the incubation of shake flasks and stock cultures. Shake flasks were incubated at room temperature and were shaken on a rotary shaker (New Brunswick Scientific Co., Inc., New Brunswick, New Jersey).

The methods employed in the identification of the experimental organism were generally those described in the Manual of Microbiological Methods (1957). All media used in biochemical tests were products of Difco Laboratories, Detroit, Michigan, or were prepared from ingredients supplied by that manufacturer. Identification of the organism was made according to Bergey's Manual of Determinative Bacteriology (Breed, Murray, and Smith, 1957).

Culture of the experimental organism. Cells used in initial studies concerning the oxidation of 2-methylalanine were harvested from Roux flasks (12 x 20 cm) containing 125 ml of 2-methylalanine medium. Larger crops of cells were obtained from the same medium dispensed into large (28 x 40 cm) stainless steel trays. The preparation of cellular extracts required larger quantities of cells than could be obtained by the above methods. The New Brunswick Continuous Culture Apparatus, Model CF-500 (New Brunswick Scientific Co., Inc.), was employed to grow gram quantities of bacteria.

The diagram shown in Figure 2 summarizes the preparation of inoculum for the continuous culture apparatus. 2-Methylalanine medium was used through Step 3 (Fig. 2) except where a liquid medium was desired, in which case the agar was omitted. The medium used in the continuous culture apparatus was a modification of 2-methylalanine medium. The content of KH_2PO_4 was increased to 0.15 per cent and 0.05 per cent of Na_2HPO_4 was added. This was done so that the pH of the medium, which rose during growth, could be controlled. Sterilization was accomplished by autoclaving the medium in concentrated form (200 ml), minus the $MgSO_4$. The $MgSO_4$, also in concentrated form, was autoclaved in another flask. The sterile concentrated medium and $MgSO_4$ solution were then added to 9.8 liters of tap water previously sterilized by autoclaving for 60 minutes.

The empty culture vessels were autoclaved for 15 minutes at 120 C, as were the stainless steel glass-wool air filters, siphon tube, and

Figure 2. Flow sheet for the preparation of inoculum for continuous culture of the experimental organism.

Step 1. Suspend growth from two slant cultures in 4 ml of sterile saline. Inoculate two Roux flasks.

Step 2. Incubate for 2-3 days at 30 C. Suspend growth in 20 ml of sterile saline.

Step 3. Inoculate two 2 liter flasks (1000 ml each). Shake for 2-3 days.

Step 4. Pitch 2 liters of inoculum into 8 liters of fresh medium in the culture vessel.

connecting tygon tubing. Ten liters of fresh medium were added to the sterile culture vessels by use of a siphon tube and air pressure from the continuous culture apparatus.

The organism was grown in the continuous culture apparatus under conditions of continuous culture. The temperature of the culture vessel was held at 30 C, while the contents were agitated (150 rpm) and aerated (approximately 1000 ml/min). Aeration was accomplished by air pressure, generated in a compressor, and forced through autoclaved glass-wool filters. Although foaming was not a problem, 1.0 to 2.0 ml of a sterile 1 to 10 dilution of foam depressant (Antifoam B, Dow Corning, Midland, Michigan) was added after inoculation.

Centrifugation of large volumes of mature culture from the continuous culture apparatus was accomplished by use of a Sharples steam driven centrifuge. The cell paste resulting from this centrifugation was suspended in 200 ml distilled water and centrifuged at $1.2 \times 10^4 \times g$ for 15 min in a Servall Model RC-2 centrifuge (Ivan Sorvall, Inc., Norwalk, Connecticut). The cells were washed an additional time by resuspension in 100 ml of 0.05 M phosphate buffer, pH 7.5. The cell paste was stored in capped centrifuge tubes at -23 C. The yield, in terms of wet weight of cells per liter of growth medium, was in the order of 0.8 to 1.0 gram.

Chemical

Materials. All inorganic salts employed in this study were

analytical reagent grade and were obtained and held only for this study. The inorganic inhibitors, sodium arsenite, potassium cyanide, and hydroxylamine were purchased from the Mallinckrodt Chemical Works, St. Louis, Missouri. Sodium azide was obtained from A. S. LaPine and Co., Chicago, Illinois.

2-Methylalanine and the other amino acids employed were products of the Nutritional Biochemical Corp., Cleveland, Ohio. 2-Methylalanine was found to yield only one ninhydrin spot when chromatographed in a number of amino acid solvent systems. Sodium pyruvate, sodium α-ketoglutarate, and oxaloacetic acid were obtained from the Nutritional Biochemical Corp. The sodium salts of α-ketobutyrate and α-ketovalerate were obtained from the Sigma Chemical Co., St. Louis, Missouri.

The compounds studied as possible intermediates of 2-methylalanine catabolism were: acetone (Mallinckrodt Chemical Works, St. Louis, Missouri), isopropylamine, isobutyric acid, 2-hydroxyisobutyric acid (Eastman Chemical Co., Rochester, New York), and methacrylic acid (K&K Laboratories, Plainview, New York).

Nutritional Biochemical Corp. was the supplier of the three inhibitors: p-chloromercuribenzoic acid, N-ethyl-maleimide, and DL-penicillamine. Iodoacetic acid and 2,4-dichlorophenol were obtained from the Eastman Chemical Co., while D-cycloserine was a gift from Eli Lilly and Co., Indianapolis, Indiana.

Pyridoxal phosphate and other derivatives of pyridoxine were purchased from the Nutritional Biochemical Corp. and from General Biochemicals, Chagrin Falls, Ohio.

Catalase, isolated from beef liver, and acylase I were purchased from the Nutritional Biochemical Corp., while L-amino acid oxidase (LAO, snake venom) was obtained from the Worthington Biochemical Corp., Freehold, New Jersey.

Other compounds and reagents, such as ethylenediaminotetraacetic acid (EDTA), tris (hydroxymethyl) aminoethane (Tris), and the reagents used in chemical assays were obtained from commercial sources.

The adsorbents employed in thin layer chromatography were obtained from Brinkmann Instruments Inc., Great Neck, New York. All materials used in column chromatography were products of Pharmacia, Uppsala, Sweden.

Assays. The disappearance of 2-methylalanine and the production of alanine, catalyzed by cellular extracts of the 2-methylalanine isolate, were measured by the ninhydrin method of Housewright and Thorne (1950). The amino acids were separated by means of thin layer chromatography on cellulose. Individual spots were scraped off the glass plates and eluted with the ninhydrin reagent. Pyruvic acid was determined by the Katsuki et al. (1961) modification of the Friedmann-Haugen method. Acetone was measured by the salicylaldehyde method of Behre (1940). Microdiffusion according to the method of Conway (1957) and titration

with standardized HCl permitted the quantitation of ammonia. Pyridoxal phosphate was estimated by the spectrophotometric method of Wada and Snell (1961).

Protein was measured by the biuret method of Gornall, Bardawill, and David (1949) when it was desirable to estimate protein in crude cellular extracts. Smaller amounts of protein were determined by the method of Lowry et al. (1951). Standard curves were prepared with crystalline bovine serum albumin (Armour Laboratories, Kankakee, Illinois).

Determinations and procedures

Manometric methods. Oxygen consumption and carbon dioxide evolution were measured manometrically with a Warburg respirator by procedures outlined by Umbreit, Burris, and Stauffer (1957). For studies involving oxygen uptake each single side-arm vessel contained 0.5 ml of 0.067 M or 0.05 M phosphate buffer (pH 7.5) with 0.2 ml of 20 per cent potassium hydroxide in the center well. A suspension of washed bacteria, standardized by relating turbidity, measured at 600 mμ in a Bausch and Lomb Spectronic 20, to a dry weight standard curve, was added to each vessel. Substrates in μmole amounts were placed in the experimental but not the control vessel, and sufficient distilled water was used to bring the volume of fluid in each vessel to 3.0 ml. Oxidation studies were carried out at 30 C with air as the gaseous phase.

The decarboxylase activity of cellular extracts was initially measured in double side-arm vessels under an atmosphere of nitrogen. When it became apparent that cellular extracts could not oxidize 2-methylalanine, the intermediates of its catabolism, or the other components of the reaction mixture, the use of nitrogen was discontinued in favor of air as the gaseous phase. All experiments were carried out at 30 C, each vessel containing 0.5 to 1.0 ml buffer, either 0.05 M phosphate or 0.05 M Tris-HCl (pH 7.8) with 0.2 ml of 10 or 20 per cent trichloroacetic acid (TCA) in a side-arm. Substrates, in μmole amounts, were placed in the other side-arm and coenzyme and experimental additions (salt, inhibitors, etc.) were added to the main compartment. The main compartment contained from 1 to 28 mg of crude or partially purified cellular extract and sufficient distilled water to make a final volume of 3.0 ml. Endogenous production of carbon dioxide was measured in a vessel not containing the methyl-substituted amino acid under study. The reaction was terminated and carbon dioxide was quantitatively released at the end of the incubation period by the addition of TCA from one of the side-arms.

Each Warburg vessel and manometer was calibrated for k_{O_2} and k_{CO_2} values at 30 C and 3.0 ml final volume by the mercury method.

All amino acids, organic acids, inhibitors, etc. were adjusted to neutral pH before addition to the reaction mixture. Solutions of keto acids and pyridoxal phosphate were prepared in 10 to 25 ml volumes,

adjusted to neutral pH, dispensed in small volumes, and held at -23 C until used. All other substrates were held at 4 C.

The preparation of 0.067 M phosphate buffer was accomplished by mixing the appropriate amount of 0.067 M KH_2PO_4 and 0.067 M $Na_2HPO_4 \cdot 2H_2O$. Other phosphate buffer was prepared by adjusting the pH of a solution of $Na_2HPO_4 \cdot 7H_2O$ to the desired pH with HCl, followed by dilution to 0.5 M. Tris-HCl buffer was prepared by the addition of an appropriate amount of 0.2 M.HCl to 50 ml of 0.2 M Tris stock solution and diluting to 200 ml. All pH determinations were carried out with the Beckman Zeromatic pH meter.

Preparation of cellular extracts. To prepare cellular extracts approximately 10 g (wet weight) of washed bacteria (fresh or frozen), a small spatula tip of fine glass beads (0.088 to 0.062 mm, Flex-O-Lite Mfg. Corp., St. Louis, Missouri), and 30 ml of 0.05 M phosphate buffer (pH 7.5) were chilled in an ice bath. The chilled slurry of bacteria was then poured into a Branson Rosett cooling cell (Rosett, 1965) and placed in an alcohol-water cold bath set at about -2 to -5 C. The bacteria were subjected to sonic disruption for 10 minutes by use of a 20 kc Branson sonifier. The temperature of the bacterial suspension remained at 10 $\pm$2 C during sonication. When larger volumes of bacteria were handled, longer periods of sonication were required. Cellular debris and abrasive were removed by centrifugation for 15 minutes at 27,000 x g in a Servall RC-2 centrifuge. The clear cellular extract was decanted and used for experimentation.

Chromatographic methods. Although one dimensional ascending paper strip chromatography was used in the early stages of this study, chromatographic separations were mainly accomplished using thin layer chromatography (TLC). Generally the methods outlined by Truter (1963) were followed. The equipment was purchased from Brinkmann Instruments, Inc.

Glass plates (20 x 20 cm) were coated with either cellulose powder (type 300G, 15 g in 90 ml distilled water) or silica gel (type G, 30 g in 60 ml distilled water). Thin films of adsorbent were spread employing an adjustable spreader (Stahl type) set routinely at 0.5 mm. The films were permitted to dry overnight at room temperature.

Samples were applied to the thin layer plates with glass tubing drawn out to fine capillaries. TLC plates were developed using the series of solvents reported in Table 1. Experimental samples of amino acids from reaction mixtures were autoclaved to drive off the TCA which had been added to precipitate protein. The 2,4-dinitrophenylhydrazones of keto acids and ketones were prepared by adding about 100 µmoles of the parent compound to 10 to 15 ml of a freshly prepared 0.2 per cent (w/v) solution of 2,4-dinitrophenylhydrazine in 2.0 N HCl. The 2,4-dinitrophenylhydrazones of experimental samples of keto acids and ketones were prepared in a similar manner. Amino acid spots were detected by spraying with a 0.25 per cent (w/v) solution of ninhydrin in acetone. Color development of the spots of the methyl-substituted

Table 1

Solvents used in thin layer chromatography

Class of compounds	Solvent	Reference
Amino acids	n-Butanol, acetic acid, water (4:1:5 v/v)	Slotta and Primosigh, 1951
	Phenol, water (3:1 w/v)	Underwood and Rockland, 1954
	n-Propanol, water (1:1 v/v)	Underwood and Rockland, 1954
	Ethanol, 25% aqueous ammonia, water (7:1:2 v/v)	Truter, 1963
Keto acids (as the 2,4-dinitrophenyl-hydrazones)	n-Butanol, ethanol, 0.5 N ammonium hydroxide (7:1:2 v/v)	El Hawary and Tompson, 1953
	t-Amyl alcohol, ethanol, water (5:1:4 v/v)	Altmann, Crook, and Datta, 1951
	Ethanol, petroleum ether (4:1 v/v)	
Ketones (as the 2,4-dinitrophenyl-hydrazones)	Ethylether, petroleum ether (5:95 v/v)	Rice, Keller, and Kirchner, 1951
	n-Heptane, methanol (96:4 v/v)	Gaddis and Ellis, 1958
	iso-Amyl alcohol, 0.25 N ammonium hydroxide (20:1 v/v)	Dancis, Hutzler, and Levitz, 1963
	Hexane, benzene, ethylether (48:48:4 v/v)	Bordet and Michel, 1963

amino acids was hastened by heating at 90 C for 5 to 15 minutes. 2,4-Dinitrophenylhydrazone derivatives were observed under ultraviolet light.

Gas chromatographic analysis of experimental reaction mixtures was performed by Herman Conrad of Micro-Tek Instruments, Inc., Baton Rouge. Acetone was qualitatively demonstrated using a Micro-Tek GC-2500R gas chromatograph equipped with a dual hydrogen flame type detector. The column (6' x 1/8", stainless steel) was packed with 10 per cent Carbowax 1000 on 80/100 Chromport XXX. Other details were detector temperature, 225 C, column temperature, 52 C, inlet temperature, 200 C, carrier gas helium,pressure, 40 psig, flow rate, 40 cc/min.

Column chromatography was carried out with dextran gels (Sephadex) according to the general methods outlined by Folin (1962) and briefly summarized in the booklet,Sephadex in Gel Filtration: Theory and Experimental Technique (1963). A Research Specialties Co. (Berkeley, California) fraction collector was used in connection with these studies.

Determination of physical characteristics of the enzyme 2-methylalanine decarboxylase. Experiments to determine the stability of the 2-methylalanine decarboxylating enzyme during the course of dialysis were carried out in various buffers, at 4 C, in cellulose dialyzer tubing. Routinely 700 to 900 volumes of buffer were employed, buffer being changed as indicated. Mixing of the buffer during dialysis was provided by mechanical stirring.

Heat inactivation experiments were performed using water baths placed on top of hot-plate-stirrers (Therm-o-mix, Precision Scientific, Chicago, Illinois). The different thermometers used were uniformly $\pm$1 C at 35 C.

Density gradient analysis was performed according to the method of Martin and Ames (1961). All manipulations were carried out at 4 C. Linear 5 to 20 per cent sucrose (w/v) gradients were constructed employing a Buchler density gradient maker (Buchler Instruments, Inc., Fort Lee, New Jersey). Centrifugation was carried out using a Spinco Model L-2 Ultracentrifuge in a SW-39 swinging bucket rotor (Beckman Instruments, Inc., Palo Alto, California). The contents of the lusteroid tubes were fractioned by punching a hole in the bottom of each tube and manually collecting drops. The sedimentation constant and molecular weight of 2-methylalanine decarboxylase were estimated using the rotor dimensions and formula reported by Martin and Ames (1961), and the enzyme catalase as an internal standard.

Enzyme purification. The purification of the bacterial enzyme or enzymes responsible for the decarboxylation of 2-methylalanine was attempted following variations around the general scheme shown below:

1. Preparation of the cellular extract (as described in this section).
2. High speed centrifugation.
3. Treatment with protamine sulfate.
4. Heat treatment.

5. Ammonium sulfate fractionation (Green and Hughes, 1955).

A more complete description of the purification protocol will be given in the Results section.

RESULTS

Isolation and identification of the experimental organism

A number of cultures capable of growing at the expense of 2-methylalanine were isolated by enrichment technique from soil. Although none of the isolates grew rapidly, the organism used for this study was chosen because of its relatively abundant growth on the isolation medium. The bacterium was aerobic, asporogenous, Gram-negative, and non-motile. It measured 0.8 to 1.1 by 1.6 to 2.3 microns when examined with the light microscope, following mounting in India ink.

The following cultural and nutritional characteristics were determined after incubation at room temperature. Growth on nutrient agar slants was filiform, smooth, and butyrous. In nutrient broth, growth was heavy throughout the tube, without formation of a pellicle or sediment. No pigment was evident in either of the above media. A brown, non-diffusible pigment was produced when the organism was cultured on a potato core. Other taxonomic biochemical reactions are summarized in Table 2.

The bacterium did not ferment any of the carbohydrates tested, as judged in phenol red broth base. Lack of fermentative ability was also demonstrated using Oxidation/Fermentation (O/F medium; Hugh and Leifson, 1953) medium overlaid with vaseline. The use of O/F medium permitted the demonstration of an oxidative attack on carbohydrates by

Table 2

Taxonomic biochemical reactions of the experimental organism

Test reaction	Determination[a] 3 days	7 days
Nitrate reduction	-	-
Indol production	-	-
Acetyl methylcarbinol production	-	-
Hydrogen sulfide production	-	-
Citrate utilization	-	-
Catalase production	+	
Oxidase production	+	
Gelatin hydrolysis	-	-[b]
Fat hydrolysis	-	-
Starch hydrolysis	+	
Cellulose hydrolysis	-	-[b]
Litmus milk	n.c.	sl. alk.
Growth on paraffin	-	-[b]
Growth on potato	sl.	+ (brown pigment)
Growth on blood serum (Loeffler's)	-	-

[a]Symbols and abbreviations: + positive, - negative, n.c. no change, sl. slight, alk. alkaline.

[b]Also negative after 14 days.

the organism. All carbohydrates tested, except lactose, were found to be oxidized. Confirmation of the results obtained with O/F medium was accomplished with cells of the 2-methylalanine isolate grown in nutrient broth and assayed manometrically for oxygen uptake. All carbohydrates tested, except lactose, were oxidized at significant rates. Ethanol was not oxidized by the organism. A summary of these results is presented in Table 3.

When the bacterium was cultured on Hydrogen sulfide, Indol, Motility (SIM) agar, Methyl Red-Voges-Proskauer (MR-VP) agar, and Triple Sugar Iron (TSI) agar, results reflecting those shown in Tables 2 and 3 were obtained.

On the basis of these observations the organism was placed in the genus Pseudomonas (Breed et al., 1957). Assignment to this genus was made after careful consideration of the possibility of placing the organism in one of the genera of the order Eubacteriales. Although the organism lacked both motility and pigmentation, which are characteristic of pseudomonads, its fundamental physiology, especially its action on carbohydrates, appeared to be similar to the members of the genus Pseudomonas. The characteristics of the organism did not fit any of the described species of Pseudomonas closely enough to permit speciation.

Development of methods for growth of the experimental organism

In order to determine the best medium for the growth of the

Table 3

Carbohydrate catabolism of the experimental organism

Carbohydrate	Fermentation[a] (acid/gas)	Oxidation	
		O/F Medium[b]	Warburg[c]
Glucose	-/-[d]	+0[e]	+
Galactose	-/-	+0	+
Fructose	-/-	+0	+
Mannose	n.p.	+0	+
Sorbose	n.p.	n.p.	+
Ribose	n.p.	+0	+
Xylose	n.p.	+0	+
Arabinose	n.p.	+0	n.p.
Rhamnose	n.p.	+0	n.p.
Sucrose	-/-	+0	+
Lactose	-/-	n.c.	-
Maltose	-/-	+0	+
Melibiose	n.p.	n.c.	n.p.
Cellobiose	n.p.	+0	n.p.
Trehalose	n.p.	+0	n.p.
Glycerol	-/-	n.p.	n.p.
Ethanol	n.p.	n.p.	-

[a]Determined in phenol red broth base during 7 days incubation.
[b]Oxidation/Fermentation Medium, determined during 7 days incubation (Hugh and Leifson, 1953).
[c]Oxygen uptake measured in the Warburg apparatus using standard technique, cells grown in nutrient broth.
[d]Symbols and abbreviations: + positive, - negative, +0 positive oxidation test, n.c. no change, n.p. not performed.
[e]Fermentative attack not observed.

organism shake flask experiments were performed in which the composition of the medium was varied. The basal salts medium of Stanier (1947) was used throughout. The effects of varying the level of 2-methylalanine and the addition of supplemental sources of nitrogen into the growth medium are shown in Table 4. The most metabolically active cells, in terms of their ability to oxidize 2-methylalanine, as measured in the Warburg apparatus, were obtained from a growth medium consisting of 0.5 per cent 2-methylalanine with no supplements. Addition of 0.005 per cent yeast extract to media containing 0.2 per cent 2-methylalanine, although decreasing the specific activity of the cells by about 28 per cent, increased the growth of the bacterium 2.7-fold. Higher levels of yeast extract markedly decreased the ability of the organism to oxidize 2-methylalanine. The inclusion of 0.5 per cent peptone into the growth medium, either in the presence or the absence of 2-methylalanine, produced cells which would not oxidize 2-methylalanine.

The effects of the addition of supplemental sources of carbon into the growth medium on the capacity of the organism to oxidize 2-methylalanine are shown in Table 5. The results of this shake flask experiment can not be directly compared with those shown in Table 4; however, the relative differences are important. It is clear that the inclusion of 0.01 per cent of sodium acetate stimulated growth appreciably but did not greatly decrease the oxidative activity of the cells toward 2-methylalanine. A growth medium was formulated by combining the best supplements as deduced from results shown in Tables 4 and 5.

Table 4

Effect of various levels of 2-methylalanine and the addition of supplemental sources of nitrogen and carbon on the growth and metabolic activity of the experimental organism

Addition[a]	Culture characteristics			
	Turbidity[b]		Δ pH[c]	Ability to oxidize 2-MA[d]
	48 hr	72 hr		
None	0.035	0.025	-0.1	---
0.1% 2-MA[e]	0.050	0.095	+0.4	5.7
0.2% 2-MA	0.040	0.115	+0.3	5.4
0.5% 2-MA	0.035	0.125	+0.3	7.5
0.2% 2-MA plus 0.005% yeast extract	0.125	0.335	+0.6	5.4
0.2% 2-MA plus 0.01% yeast extract	0.173	0.335	+0.8	2.7
0.2% 2-MA plus 0.025% yeast extract	0.275	0.355	+0.9	2.0
0.2% 2-MA plus 0.5% peptone	1,200	-----	+1.3	0.3
0.5% peptone	1.200	-----	+1.3	0.0
0.2% 2-MA[f]	0.040	0.110	+0.6	4.4
0.2% 2-MA plus 0.01% yeast extract[f]	0.195	0.355	+1.0	3.7

[a]Added to basal salts (Stanier, 1947) .
[b]Measured at 600 mμ.
[c]Initial pH 7.1.
[d]Measured in terms of μmoles of oxygen uptake per hour per 0.5 ml of cells adjusted to an absorbancy of 0.30 at 600 mμ.
[e]2-MA is 2-methylalanine.
[f]2-Methylalanine as the only added nitrogen source.

Table 5

Effect of the addition of supplemental sources of carbon on the growth and metabolic activity of the experimental organism

Addition[a]	Culture characteristics		
	Turbidity[b] 48 hr	Δ pH[c]	Ability to oxidize 2-MA[d]
None	0.030	0.0	---
0.2% 2-MA[e]	0.075	+0.3	6.1
0.2% 2-MA plus 0.01% sodium acetate	0.200	+0.5	5.6
0.2% 2-MA plus 0.05% sodium acetate	0.355	+0.7	4.9
0.2% 2-MA plus 0.07% sodium acetate	0.320	+0.7	2.4
0.2% 2-MA plus 0.01% sodium formate	0.070	+0.1	1.3
0.2% 2-MA plus 0.02% glucose	0.360	+0.3	3.5

a,b,c,d,e Identical to those described in Table 4.

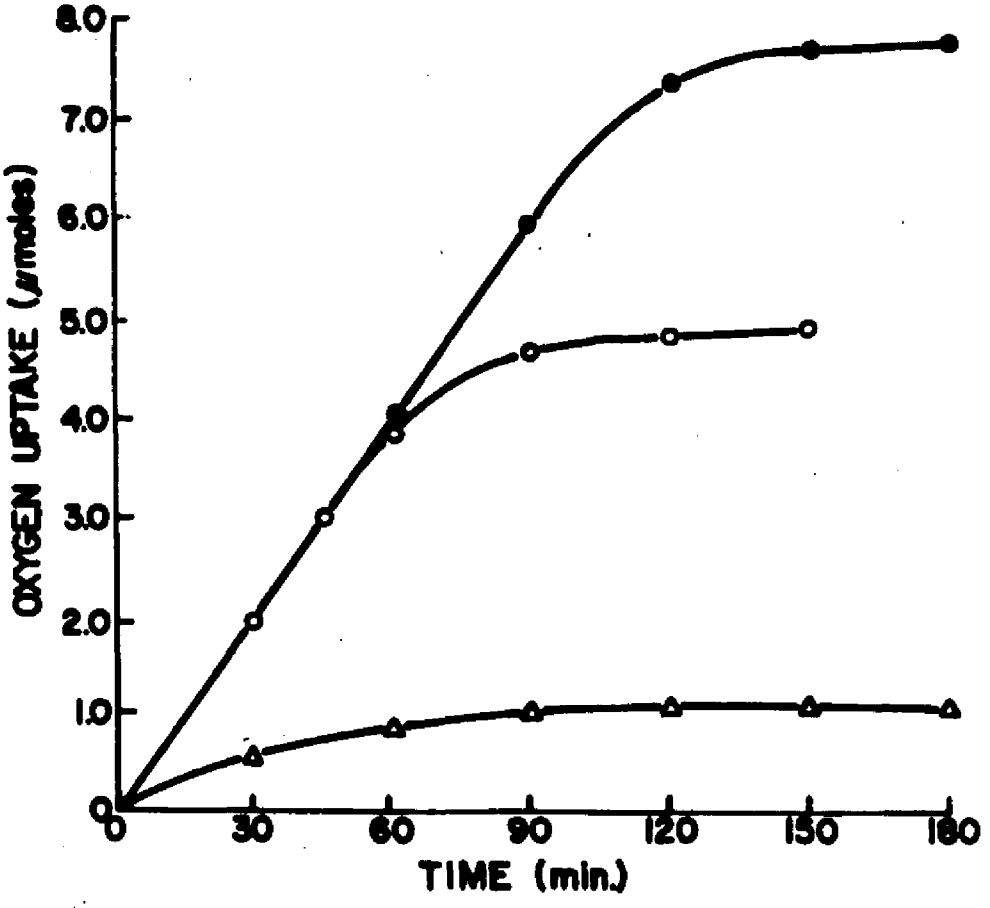
That is, yeast extract and sodium acetate, each at a concentration of 0.005 per cent, when added to 0.2 per cent 2-methylalanine in the basal salts solution were found to permit abundant growth of bacteria which were able to rapidly oxidize 2-methylalanine.

Additional shake flask experiments indicated that adjusting the initial pH of the growth medium to 7.0 to 7.2 gave the best growth of cells which possessed the greatest activity on 2-methylalanine. A similar experiment showed that while growth did not attain its maximum point until 72 hours after inoculation, harvested cells reached their maximum ability to oxidize 2-methylalanine as early as 20 hours after inoculation.

Demonstration of the oxidation of 2-methylalanine by whole cells

When suspensions of washed whole cells of the bacterium were incubated with limiting quantities of 2-methylalanine in a Warburg apparatus, oxygen uptake was found to be 60 to 65 per cent of the theoretical value. These data (Fig. 3) indicate the ability of the organism to completely oxidize the amino acid. Oxidative assimilation may account for the discrepancy between the experimental results and the theoretical oxygen utilization. Addition of 2,4-dinitrophenol at a concentration of 10^{-5}M increased the oxygen uptake to approximately 75 per cent of theoretical. The optimum pH for the oxidation of 2-methylalanine by whole cells was between 7.0 and 7.5, with no sharp peak discernable.

Figure 3. Oxidation of 2-methylalanine. Each Warburg vessel contained 15.0 mg (dry weight) of cells; 0.5 ml of 0.067 M phosphate buffer (pH 7.0); 1 μmole (○), 2 μmole (●), or no (△) 2-methylalanine; 0.2 ml of KOH in the center well; and distilled water to a final volume of 3.0 ml.


8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
OXYGEN UPTAKE (μmoles)
0
30
60
90
120
150
180
TIME (min.)

Demonstration of the oxidation of intermediates in the dissimilation of 2-methylalanine and the adaptive nature of the system

The ability of the organism to oxidize 2-methylalanine is adaptive. When cultivated on a medium containing glucose or peptone (Tables 4 and 5), or both, the ability of the bacterium to oxidize 2-methylalanine was lost or greatly depressed; this effect was noticed even if the inducer was incorporated into the media. However, it was observed that cells grown on medium containing glucose or isobutyric acid as a sole source of carbon would adapt to 2-methylalanine. This was accomplished by shaking a heavy suspension of glucose- or isobutyrate-grown cells in the presence of 2-methylalanine for an appropriate period of time.

The results shown in Table 6 illustrate the adaptive nature of 2-methylalanine dissimilation by this organism. When the organism was grown on a medium containing 2-methylalanine as a sole source of carbon, very active cells were obtained; however, growth was sparse. Inclusion of a trace amount of sodium acetate markedly stimulated growth and produced cells active toward 2-methylalanine (see also Table 5). Acetate-grown cells oxidized 2-methylalanine very slowly.

Table 6 also presents data concerning the ability of resting cells to oxidize possible intermediates in the dissimilation of 2-methylalanine. These compounds were chosen as possible intermediates after a consideration of the feasible pathways by which the 2-methylalanine molecule might be attacked (Fig. 1). The rate at which acetone was oxidized by the cells grown on 2-methylalanine suggested

Table 6

Oxidation of 2-methylalanine and possible intermediates in the dissimilation of 2-methylalanine by whole cells grown on different media[a]

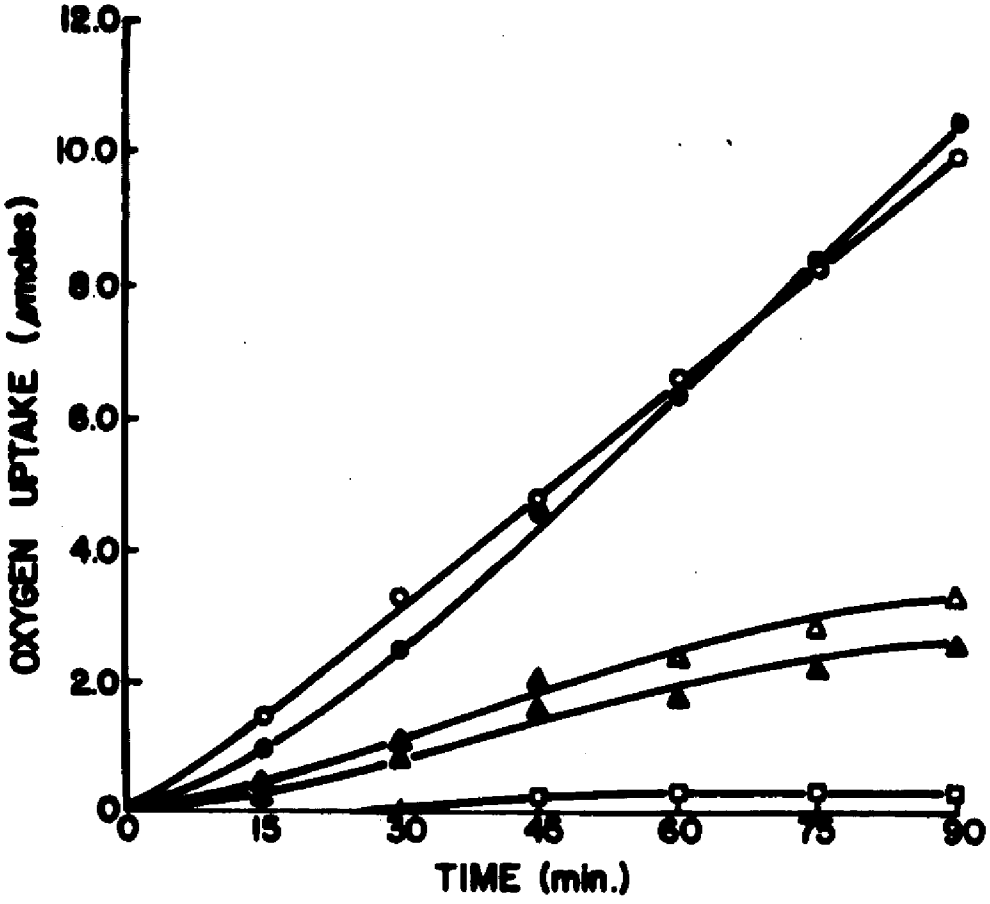
Substrate	Growth media[b] 1	2	3
2-Methylalanine	18.3[c]	17.6	2.3
Isopropylamine	1.3	2.7	2.0
Acetone	23.2	23.4	1.9
Isobutyrate	5.0	4.0	4.3
2-Hydroxyisobutyrate	not done	2.3	1.9
Methacrylate	not done	3.7	9.1
2-Methylserine	7.2	5.9	1.9
Endogenous	1.2	2.0	1.8

[a]Conditions identical to those described in Figure 3, except that the substrate concentration was 12.5 μmoles.
[b]Growth media contained basal salts plus (1) 0.2% 2-methylalanine, (2) 0.2% 2-methylalanine plus 0.005% sodium acetate, (3) 0.2% sodium acetate.
[c]Figures indicate oxygen uptake expressed as μmoles per hour.

that this compound was an intermediate. However, since isopropylamine was not oxidized, direct decarboxylation of 2-methylalanine to isopropylamine did not seem likely. It also appeared unlikely that a reductive or desaturative deamination was operative, since isobutyric acid and methacrylic acid were oxidized to an equal or greater extent by non-adapted cells than by adapted cells. The fact that 2-hydroxyisobutyric acid was very slowly oxidized by adapted cells seemed to exclude this compound as an intermediate. 2-Methylserine could be an intermediate if oxidative attack were to occur on a methyl group of 2-methylalanine. Although a pathway through 2-methylserine could not be discounted at this point, these data (Table 6) indicated that acetone was an intermediate in the catabolism of 2-methylalanine. Moreover, since isopropylamine was not oxidized, the conversion of 2-methylalanine to acetone appeared to proceed by way of a decarboxylation dependent transamination (Fig. 1, pathway 2).

The specificity of the 2-methylalanine oxidizing enzyme system was determined by utilizing different 2-methyl amino acids as substrates. The rate at which whole cells grown on 2-methylalanine medium oxidized the amino acids is shown in Figure 4. Isovaline (DL-2-methyl-2-aminobutyric acid), a homolog of 2-methylalanine, was oxidized at a rate equal to that of 2-methylalanine. It is obvious that the chemical character of the amino acid residue or side chain is of considerable importance, since the organism's ability to oxidize

Figure 4. Oxidation of 2-methyl amino acids by whole cells. Each Warburg vessel contained 25.0 mg (dry weight) of cells, 0.5 ml of 0.067 M phosphate buffer (pH 7.5), 12.5 μmoles of substrate, 0.2 ml of 20% KOH in the center well, and distilled water to a total volume of 3.0 ml. Symbols: 2-methylalanine (○), isovaline (●), 2-methylserine (△), 2-methylmethionine (▲), 2-methylglutamic acid (□). All data minus endogenous activity, which was 1.6 μmoles after 90 min.


12.0
10.0
8.0
6.0
4.0
2.0
0
OXYGEN UPTAKE (μmoles)
0
15
30
45
60
75
90
TIME (min.)

the different 2-methyl amino acids decreased as the character of the residue changed from that of a hydrocarbon, to a hydroxymethyl, to a thioether, to an acid. In an experiment in which limiting amounts of D-, L-, and DL-isovaline were incubated with whole cells grown on 2-methylalanine, oxidation of both isomers was observed (Table 7). DL-Isovaline was resolved by the method of Baker et al. (1952) and Baker and Sober (1953). This was accomplished by the formation of the N-chloroacetyl derivative of DL-isovaline and subsequent specific hydrolysis by the enzyme, acylase I. Free L-isovaline was separated from N-chloroacetyl-D-isovaline on a Dowex 50 ion exchange column. The optical rotation for the isolated isomers was: $[\alpha]^{25}$D-10.11, and L-isovaline $[\alpha]^{25}$D+10.25 ($[\alpha]^{25}$D-11.28, and $[\alpha]^{25}$D+11.13, respectively, Baker et al., 1952).

Demonstration of the decarboxylation of 2-methylalanine by cellular extracts

Cell permeability barriers could explain the inability of the organism to oxidize possible intermediates, such as isopropylamine; therefore, cellular extracts were prepared from cells grown in 2-methyl-alanine medium. Isopropylamine was not oxidized by cellular extracts prepared by a variety of methods (alumina grinding, French pressure cell treatment, sonication). Likewise, initial attempts with cellular extracts failed to demonstrate significant carbon dioxide production under aerobic or anaerobic conditions. It had been observed that whole

Table 7

Oxidation of D-, L-, and DL-isovaline by whole cells[a]

	Endogenous	D-isomer	L-isomer	DL-mixture
μmoles of oxygen taken up in 120 min	15.3	23.5	24.6	24.8
Minus endogenous oxygen uptake	----	8.2	9.3	9.5
Per cent of theoretical	----	68.0	77.0	79.0

[a]Conditions identical to those described in Figure 3, except that 2.0 μmoles of D-, L-, or DL-isovaline were added as substrate.

cells could produce carbon dioxide when incubated with 2-methylalanine in an aerobic atmosphere. No carbon dioxide was produced when whole cells were placed under a nitrogen atmosphere.

If a decarboxylation dependent transamination (Fig. 1, pathway 2) was operative, as the first step in the catabolism of 2-methylalanine, a method for regeneration of pyridoxal phosphate should allow activity by cellular extracts (15 to 28 mg/ml). Experimental data obtained when crude cellular extracts prepared by sonication (at 4 C) were incubated in the presence of 2-methylalanine, pyridoxal phosphate, and pyruvate, are shown in Table 8. A dependence on pyridoxal phosphate was observed in dialyzed cellular extracts. Moreover, the inclusion of a keto acid was an absolute requirement. Table 8 also indicates that dialysis (against 5×10^{-5} M phosphate buffer, pH 7.0, for 12 hours) stimulated the system. In summary, the data reported in Table 8 show that an active 2-methylalanine decarboxylating system can be demonstrated, providing a keto acid was present. It is also evident that pyridoxal phosphate was the coenzyme, since carbon dioxide produced in the dialyzed preparation minus pyridoxal phosphate was one half that of the undialyzed preparation. Further evidence for the role of pyridoxal phosphate as coenzyme is shown by the production of 6 times as much carbon dioxide by the complete dialyzed system when compared to the dialyzed system without added pyridoxal phosphate. Pyruvate apparently permits the regeneration of pyridoxal phosphate from pyridoxamine phosphate. Apparently

Table 8

Carbon dioxide production by cellular extracts

Experiment	System[a] Complete	Minus pyridoxal phosphate	Minus pyruvate
1. Undialyzed	2.7[b]	2.0	not done
Dialyzed	5.8	1.0	not done
2. Undialyzed	0.2	not done	0.0
Dialyzed	2.0	not done	0.1

[a]Complete reaction mixture contained 12.5 μmoles of 2-methylalanine, 12.5 μmoles of sodium pyruvate, 150 μg of pyridoxal phosphate, 0.5 ml of 0.067 M phosphate buffer (pH 7.0), 1.0 ml of cellular extract, and distilled water to a final volume of 3.0 ml. The reaction was stopped by the addition of 0.2 ml of 20% TCA and carbon dioxide measured manometrically. The experiment was carried out under a nitrogen atmosphere.

[b]Figures indicate amount of carbon dioxide expressed in microliters per milligram of protein per hour.

dialysis removes an inhibitory factor, possibly a metal ion, since carbon dioxide production was greater in the complete dialyzed system than in the complete undialyzed system. Dialysis at 4 C for 12 hours was routinely used in the following experiments unless mentioned otherwise.

The optimum pH (Fig. 5) for the decarboxylation of 2-methylalanine by the cellular extract was found to be 7.8. The carbon dioxide produced by the cellular extract at the optimal pH represents decarboxylation of 62 per cent of the added substrate.

The data shown in Figure 4 are apparently not related to cellular permeability characteristics, because data pertaining to the decarboxylase activity of cellular extracts on the different 2-methyl amino acids show the same relative rates (Table 9). The rate of carbon dioxide production from DL-isovaline was greater than that of 2-methylalanine, strongly suggesting that isovaline was a more suitable substrate. In preliminary experiments it was also determined that L-isovaline was decarboxylated at approximately four times the rate of D-isovaline. The fact that carbon dioxide was produced by cellular extracts from four of the six 2-methyl amino acids tested as substrates indicates a lack of absolute specificity.

Thin layer chromatography of the contents of Warburg vessels in which decarboxylation of 2-methylalanine had been demonstrated revealed two principal products. The production of alanine was demonstrated in numerous solvent systems suitable for amino acids (Table 10); no isopropylamine was detected. Isopropylamine was not detected,

Figure 5. Effect of pH on the decarboxylation of 2-methylalanine by cellular extracts. The reaction mixture was identical to that described in Table 8, except that 1.0 ml of 0.2 M phosphate buffer of the desired pH was used. The experiment was stopped after 120 min.

CARBON DIOXIDE (μmoles)
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
pH
6.5
6.0
5.5
7.0
7.5
8.0
8.5

Table 9

Comparison of the decarboxylase activity of cellular extracts and the oxidative activity of whole cells toward 2-methyl amino acids

Substrate	Carbon dioxide produced[a]	Oxygen uptake[b]
2-Methylalanine	2.0	9.9
Isovaline	7.0	10.4
2-Methylserine	0.9	3.2
2-Methylmethionine	0.5	2.5
2-Methylglutamate	0.0	0.3
2-Methyldihydroxyphenylalanine	0.0	c

[a]Expressed as μmoles CO_2 per 120 min. Conditions identical to those described in Table 8, except that phosphate buffer pH 7.8 was used.

[b]Expressed as μmoles oxygen uptake per 90 min. Conditions identical to those described in Figure 4.

[c]Auto-oxidized.

Table 10

Rf values of amino acids

Amino acid	Rf in solvent[a] 1[b] A	1[b] B	2 A	2 B	3 A	4 A
Alanine	0.28	0.12	0.60	0.21	0.65	0.67
Aspartic acid	0.12	0.08	0.12	----	0.56	0.39
2-Methylalanine	0.37	0.19	0.70	0.25	0.72	0.72
Isovaline	0.50	0.25	0.80	0.31	0.77	0.77
2-Methylserine	0.26	0.14	0.53	0.17	0.66	0.72
2-Methylglutamic acid	0.28	0.17	0.39	0.08	0.61	0.51
2-Methylmethionine	0.62	0.33	0.82	0.38	0.82	0.78
2-Methyldihydroxy-phenylalanine	0.39	----	0.55	0.25	0.73	----
Isopropylamine[c]	0.57	----	----	----	----	----

[a]The solvents were:
No. 1. Butanol, acetic acid, water
2. Phenol, water
3. Propanol, water
4. Ethanol, ammonium hydroxide, water
See Materials and Methods section for details.

[b]Plates made of cellulose powder (A) or silica gel (B).

[c]Gave brown spot.

using paper strip chromatography (Rfs not shown in Table 10). The Rf values of all the 2-methyl amino acids used in this study are listed in Table 10 in order to characterize their properties on thin layer chromotoplates. The second product, acetone, and residual pyruvate were chromatographically detected by use of methods specific for keto compounds (Table 11). Acetone was also detected by gas chromatography using a column packed with 10 per cent Carbowax 1000.

In order to determine the specificity of the keto acid requirement for the 2-methylalanine decarboxylating enzyme system, an experiment was performed with different keto acids (Table 12). All keto acids were sodium salts except for oxaloacetic acid which was adjusted to neutrality with sodium hydroxide before use. The data shown in Table 12 reflect enzyme activities greater than those previously obtained. The more active cellular extracts may be related to the development of improved techniques in their preparation. It is clear from the data presented in Table 12 that α-ketobutyrate serves as well as pyruvate as an amino acid acceptor in the coupled decarboxylation and transamination of 2-methylalanine. Thin layer chromatography of the amino acid product of the reaction mixture in which α-ketobutyrate served as a keto acid revealed spots with Rf values identical to 2-methylalanine. However, since 2-methylalanine and 2-aminobutyric acid showed the same Rf values in the solvent systems used, it was assumed that 2-aminobutyric acid was formed. α-Ketovalerate served only a third as well

Table 11

Rf values of 2,4-dinitrophenylhydrazones of keto acids and ketones

Parent compound	Rf in solvents[a] 1[b] A	2 A	3 A
Pyruvate	0.28	0.44	0.53
Oxaloacetate	0.18	0.14	0.12
α-Ketoglutarate	0.03	0.13	0.04

Parent compound	4 A	5 B	6 A	7 A
Acetone	0.12	0.58	0.57	0.25
Butanone	0.21	0.74	0.61	0.37

[a]The solvents were:

No. 1. Butanol, ethanol, ammonium hydroxide
2. t-Amyl alcohol, ethanol, water
3. Ethanol, petroleum ether
4. Ethylether, petroleum ether
5. n-Heptane, methanol
6. iso-Amyl alcohol, ammonium hydroxide
7. Hexane, benzene, ethylether

See Materials and Methods section for details.

[b]Plates made of silica gel (A) and cellulose powder (B).

Table 12

Ability of different keto acids to serve as amino group acceptors in the decarboxylation of 2-methylalanine.

Keto acid	Activity[a]
Pyruvate	7.8[b]
α-Ketobutyrate	7.5
α-Ketovalerate	2.0
α-Ketoglutarate	0.0
Oxaloacetate	2.6[c]

[a]Assay conditions identical to those described in Table 8 except that 0.2 ml of 0.2 M phosphate buffer (pH 7.8) was used and the keto acids varied as shown.

[b]Expressed as μmoles CO_2 per hour.

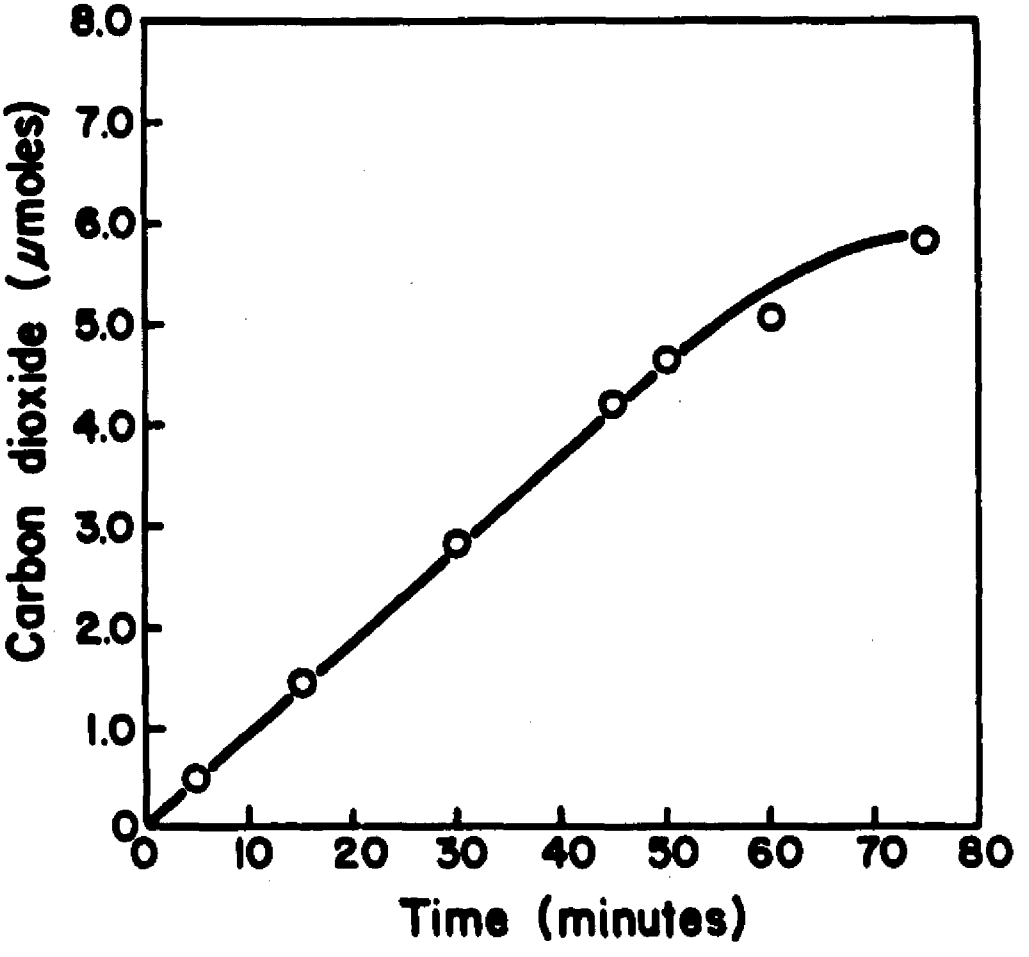
[c]Activity due to decarboxylation of oxaloacetate to pyruvate (as shown in control experiment) with the resulting pyruvate acting in the decarboxylation of 2-methylalanine.

as pyruvate; 2-aminovalerate (norvaline) was chromatographically detected. Oxaloacetate and α-ketoglutarate did not serve as an amino group acceptor in this system. Analysis of the reaction mixture in which oxaloacetate was present showed alanine formation (see Table 12) due to production of pyruvate from oxaloacetate.

All preceding experiments with cellular extracts were carried out under an atmosphere of nitrogen. Since the exchange of the atmosphere in the Warburg vessels required an added step in the manometric assay, the use of an air phase was studied. 2-Methylalanine was found to be decarboxylated at the same rate under an air atmosphere as under the nitrogen atmosphere. Moreover, it was found that neither undialyzed or dialyzed cellular extracts could oxidize 2-methylalanine, pyruvate, or acetone. The cellular extracts did not decarboxylate pyruvate. Since the use of a nitrogen atmosphere was not necessary, all following experiments were carried out under an air atmosphere, except the study of the stoichiometry of the reaction, which was performed before the above experiments were completed.

Figure 6 demonstrated that the decarboxylation of 2-methylalanine was linear with time for the first 60 min under the conditions of assay. It was also determined that Tris-HCl buffer (pH 7.8) at final molar concentration of 0.05 to 0.2 would serve as well as or better than phosphate buffer.

Figure 6. Rate of carbon dioxide production from 2-methylalanine by cellular extracts. Reaction mixture contained 20 μmoles of 2-methylalanine, 20 μmoles of sodium pyruvate, 0.5 μmole of pyridoxal phosphate, 0.2 ml of 0.2 M phosphate buffer (pH 7.8), 2 μmoles of EDTA, 0.5 ml of cellular extract, and distilled water to 3.0 ml. At intervals of time 20% TCA was tipped in from a side arm to stop the reaction and permit quantitative release of carbon dioxide.


Carbon dioxide (μmoles)
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
0
10
20
30
40
50
60
70
80
Time (minutes)

Stoichiometry of the decarboxylation of 2-methylalanine

To delineate more carefully the catabolism of 2-methylalanine an experiment was performed to determine the stoichiometry of the reaction. The reaction was carried out in a Warburg vessel as is described in Table 13. After termination of the reaction with acid and measurement of the carbon dioxide produced, the reaction mixture was centrifuged. The supernatant fluid was analyzed for the disappearance of the starting constituents and the production of acetone, alanine, and ammonia. The data shown (Table 13) were corrected for endogenous activity by subtracting appropriate values obtained from controls consisting of the complete system plus inactivated cellular extract (boiled 5 min) and the complete system minus 2-methylalanine. The results from the complete system offer a reasonable balance, in view of the fact that a crude cellular extract was employed as the source of enzyme. Although more 2-methylalanine disappeared than could be accounted for, a difference of about 0.9 μmoles, it may be seen that values of 2.0 μmoles of carbon dioxide, 1.6 μmoles of acetone, and 2.2 μmoles of amino nitrogen (alanine plus ammonia) roughly balance. Isopropylamine was not detected. The results obtained when pyruvate or pyridoxal phospate were omitted again indicated the dependence of the decarboxylating enzyme or enzymes on the compounds.

An attempt was made to assay directly for the disappearance of pyridoxal phosphate in the 2-methylalanine decarboxylating system.

Table 13

Stoichiometry of the decarboxylation dependent transamination of 2-methylalanine

System[a]	Change in reactants and products[b]					
	2-Methyl-alanine	Pyruvate	Carbon dioxide	Acetone	Amino nitrogen Alanine	Amino nitrogen Ammonia
1a. Complete	-3.1	-4.3	+3.5	+1.6	+3.0	+2.8
1b. Complete with boiled enzyme	-0.2	-0.1	+0.5	0.0	+1.2	+2.1
1c. Complete minus 2-methylalanine	----	-1.1	+1.5	0.0	+1.3	+2.1
1d. Complete corrected for endogenous	-2.9	-3.2	+2.0	+1.6	+1.7	+0.7
2. Complete minus pyridoxal phosphate[c]	-1.2	-1.3	+0.6	+0.5	+0.1	+0.3
3. Complete minus pyruvate[c]	-1.4	----	+0.4	+0.7	+0.3	+0.2

[a]Complete reaction mixture contained 20.0 μmoles of 2-methylalanine, 20.0 μmoles of sodium pyruvate, 150 μg of pyridoxal phosphate, 0.5 ml of 0.2 M phosphate buffer (pH 7.8), 1.0 ml of cellular extract, and distilled water to a final volume of 3.0 ml. The reaction was carried out under a nitrogen atmosphere, and was terminated at the end of 120 min by the addition of 0.2 ml of 20% metaphosphoric acid. Carbon dioxide was measured manometrically. For other analytical methods, see Materials and Methods.

[b]Expressed in μmoles.

[c]These data were corrected by appropriate controls.

Even when concentrations of pyridoxal phosphate approaching stoichiometric amounts were used, no measurable change could be detected by the spectrophotometric method of Wada and Snell (1961). When carbon dioxide production was measured as an index of activity, it was found that catalytic levels of pyruvate could stimulate the decarboxylation of 2-methylalanine. A 2-fold increase was observed in numerous experiments when such a system was compared to an identical experiment minus the pyruvate. It was observed, for example, that 0.58 μmoles of carbon dioxide was produced in one hour by a dialyzed cellular extract in the presence of 5.0 μmoles of pyridoxal phosphate, while a value of 1.02 was obtained in the same system when 0.2 μmoles of sodium pyruvate were added. In no case, however, could the disappearance of pyridoxal phosphate be correlated with this activity. Since a crude cellular extract was employed in these studies, the effect of pyruvate may be explained by the action of transaminases which could regenerate low levels of the keto acid.

Pyridoxamine phosphate was found to be two-thirds as effective as pyridoxal phosphate for activation of the enzyme system.

At this point the descriptive name 2-methylalanine decarboxylase was applied to the enzyme or enzymes responsible for the decarboxylation of 2-methylalanine.

Effect of inhibitors and divalent cations on the decarboxylation of 2-methylalanine

Since dialysis was found to stimulate the decarboxylation of 2-methylalanine by cellular extracts, other methods of removing low molecular weight elements (cations) were investigated. Passage of crude cellular extracts through a column (2 x 15 cm) containing Sephadex G-25 dextran gel resulted in a stimulation similar to that obtained by dialysis. This stimulation, however, was inconsistent, as was treatment of extracts by dialysis.

Treatment of crude cellular extracts with the sodium salt of the chelating agent EDTA (pH 7.5) resulted in a stimulation at low levels (2.0 μmoles/ml reaction mixture) and inhibition at high concentrations (10.0 to 20.0 μmoles/ml reaction mixture). EDTA neutralized with Tris did not show an inhibitory effect when added at a concentration of 15.0 μmoles/ml of reaction mixture, but did stimulate, as did sodium EDTA, at lower concentrations. The sodium salt of EDTA neither stimulated nor inhibited a crude 2-methylalanine decarboxylase which had been dialyzed against phosphate buffer containing 0.2 M KCl.

If removal of divalent cations by dialysis, Sephadex gel, or treatment with EDTA stimulate the enzyme, it should be possible to show an inhibitory effect of such ions. This was accomplished, as is shown in Table 14. The cellular extract was dialyzed against 10^{-5}M phosphate buffer (pH 7.5) containing 0.2 M KCl for 10 hours prior to use. The dialyzed cellular extract was inhibited to some extent by all of the

Table 14

Effect of divalent cations on 2-methylalanine decarboxylase[a]

Molar concentration	Metals[b]					
	Magnesium[c]	Nickel	Cobalt	Zinc	Manganese	Calcium
1×10^{-3}	39.4[d]	22.8	30.0	---	5.8	15.5
1×10^{-4}	24.5	10.2	6.4	3.0	5.0	11.2
1×10^{-5}	1.0	12.5	8.8	---	2.0	5.5

[a]Assayed as described in Figure 6, except that no EDTA was added.

[b]All cations added as sulfate salts except calcium and zinc which were chloride salts.

[c]Arranged by increasing ionic radius (Moeller, 1958), left to right.

[d]Expressed as per cent inhibition of enzyme activity with no divalent cation added.

cations shown, but most drastically by magnesium. The decrease in the degree of inhibition may be roughly correlated to the increase in ionic radius of each of the metal ions. This was best seen when the metal ion concentration was 1×10^{-4} M. However, these data only suggest that the effect of the metal ion may be related to the ionic size.

The effects of various inhibitors on the enzyme system are shown in Table 15. Cyanide and hydroxylamine, both carbonyl reagents, markedly inhibited the enzyme. This was most likely due to reaction with the formyl group of pyridoxal phosphate. The inhibitory effect of cyanide does not appear to be related to energy metabolism since sodium azide was not inhibitory. Sodium arsenite was strongly inhibitory; its mode of inhibition is not clear. The enzyme does not appear to be sulfhydryl group dependent since *p*-chloromercuribenzoic acid and N-ethyl-maleimide were not inhibitory; iodoacetic acid inhibited slightly, perhaps due to the alkylation of an amino acid at the catalytic site on the enzyme. Inhibitors characteristically known to inhibit pyridoxal phosphate dependent enzymes, such as D-cycloserine, DL-penicillamine, and L-cysteine, strongly inhibited the decarboxylation of 2-methylalanine by cellular extracts.

Effect of dialysis on crude 2-methylalanine decarboxylase

It has been pointed out that the dialysis of cellular extracts was required to obtain an active preparation. However, during

Table 15

Effect of inhibitors on 2-methylalanine decarboxylase[a]

Inhibitor	Concentration	
	1×10^{-3} M	5×10^{-4} M
None	0.0[b]	----
Potassium cyanide	72.3	46.0
Hydroxylamine	----	100.0
Sodium azide	----	0.0
Sodium arsenite	79.0	81.2
ρ-Chloromercuribenzoic acid	----	3.1
N-Ethyl-maleimide	0.0	0.0
Iodoacetic acid	15.5	----
D-Cycloserine	75.6	----
DL-Penicillamine	72.3	----
L-Cysteine	19.6	----

[a] Assayed as described in Figure 6, except that no EDTA was added.

[b] Expressed as per cent inhibition of enzyme activity with no inhibitor added.

subsequent experiments, employing either dialysis or Sephadex gel filtration to desalt the cellular extracts, enzyme activity was often found to be diminished rather than stimulated. This inconsistency led to examining dialysis of the enzyme more carefully. Figure 7 shows the marked inactivation of the enzyme by dialysis against 10^{-4} M phosphate buffer (pH 7.5). Specific activity rapidly decreased during the first nine hours of dialysis and was only 15 per cent of the initial value after 21 hours. Addition of mercaptoethanol to 10^{-3} M provided little protection. The addition of 10^{-4} M 2-methylalanine, 10^{-5} M pyridoxal phosphate, or adenosine monophosphate (AMP) at 10^{-4} M, did not stabilize the enzyme during dialysis. Addition of AMP and glutathione together or separately to dialyzed preparations had no reactivating effect.

A sample of the mercaptoethanol-free material dialyzed for 21 hours (Fig. 7) can be reactivated to some degree as is shown in Table 16. Some degree of reactivation was obtained with boiled extract (5 min) and identical reactivation was obtained with ashed extract resuspended to the original volume. A greater degree of reactivation, produced by making the reaction mixture 0.1 M with respect to KCl, indicated that the diminution of ionic strength was perhaps the cause of loss of enzyme activity during dialysis.

The data graphed in Figure 8 show that sufficient levels of KCl protected the enzyme during dialysis against 10^{-4} M phosphate

Figure 7. Effect of dialysis against dilute phosphate buffer with and without mercaptoethanol. 2-Methylalanine decarboxylase activity was assayed as shown in Figure 6, except that no EDTA was used, the level of pyridoxal phosphate was 0.25 μmoles, and 0.5 ml of 0.05 M Tris-HCl buffer (pH 7.8) was used. Specific activity was defined as μmoles CO_2 per mg protein per 60 min. Symbols: with 10^{-3}M mercaptoethanol (△) and without mercaptoethanol (○), b.c. refers to buffer changed.

Figure 8. Effect of different levels of KCl during dialysis against dilute phosphate buffer. Enzyme assay and definition of specific activity are as shown in Figure 7. Symbols: no added KCl (●), 0.05 M KCl (□), 0.10 M KCL (△), and 0.20 M KCl (○). Molar concentrations are final buffer concentrations.

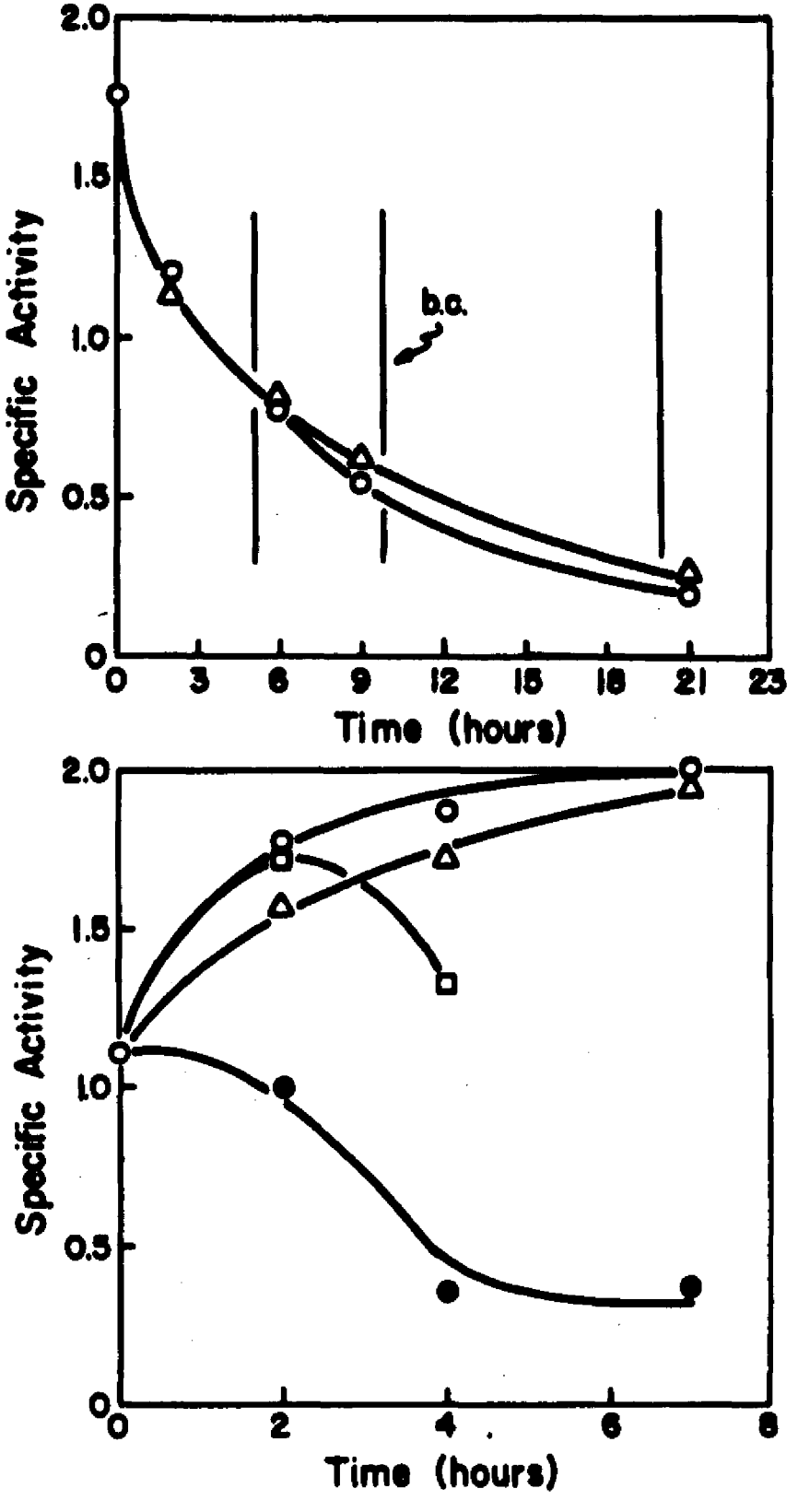

2.0
1.5
1.0
0.5
0
Specific Activity
b.a.
0
3
6
9
12
15
18
21
23
Time (hours)
2.0
1.5
1.0
0.5
0
Specific Activity
0
2
4
6
8
Time (hours)

Table 16

Partial reactivation of 2-methylalanine decarboxylase inactivated by dialysis

System	Specific activity[a]	Per cent activity
1. Undialyzed preparation[b]	1.75	100.0
2. Enzyme dialyzed 21 hr[b]	0.27	15.5
3. No. 2 plus boiled No. 1	0.37	21.2
4. No. 2 plus ashed No. 1	0.36	20.6
5. No. 2 plus 0.1 M KCl	0.41	23.5

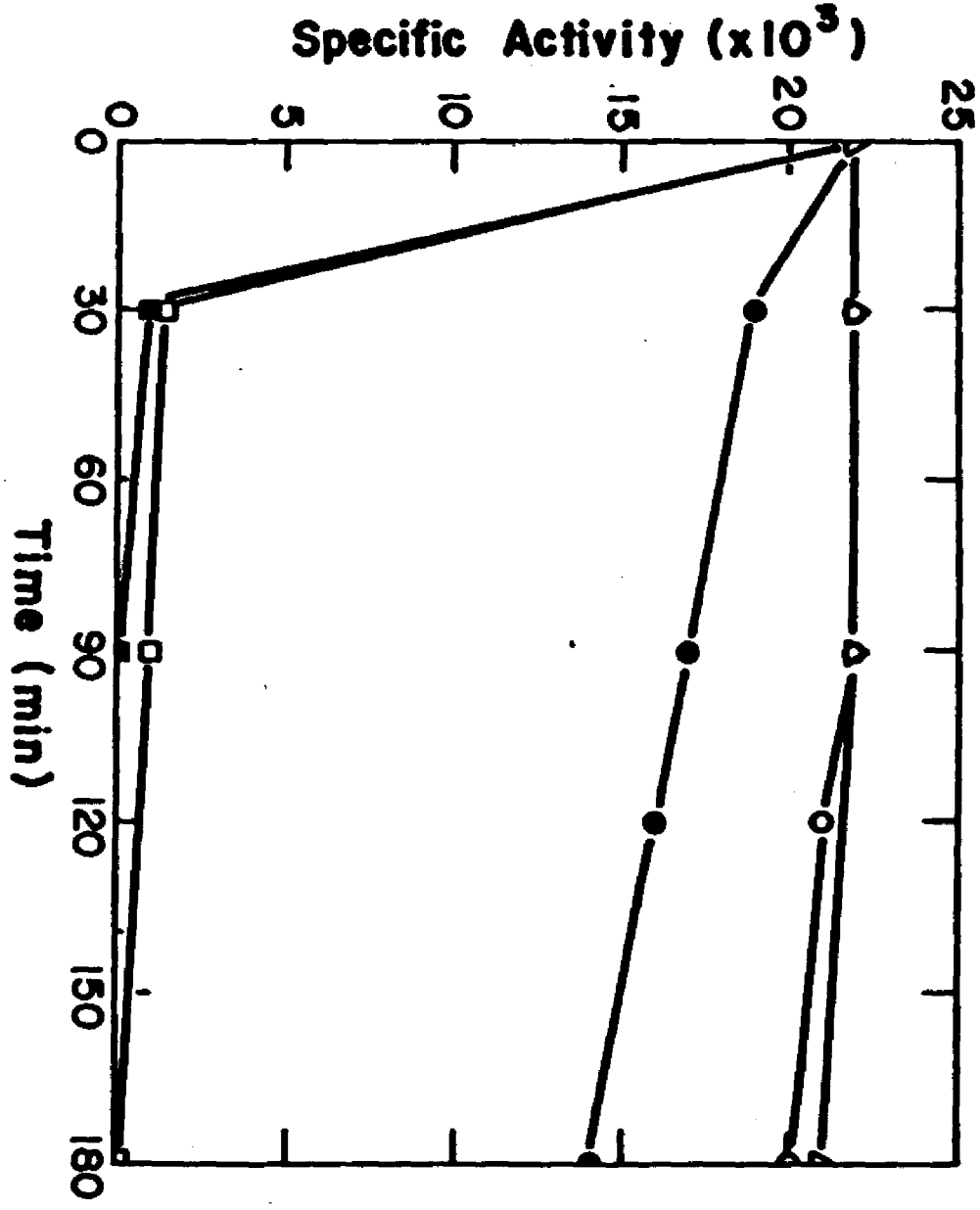
[a]Specific activity defined as μmoles CO_2 per mg protein per hour. Enzyme assayed as described in Figure 7.

[b]Same enzyme preparation used in Figure 7.

buffer (pH 7.5) and permitted the demonstration of the stimulatory effect of dialysis. Apparently 0.05 M KCl, although showing the stimulatory effect, was not sufficient to protect against inactivation by dialysis longer than three hours. Attempts were made to reactivate enzyme which had been shocked by diminution of ionic strength by passage through a Sephadex G-25 column, equilibrated against 10^{-5} M phosphate buffer, using from 0.1 to 1.0 M (final concentration) KCl. Reactivation experiments were incubated at 4 C for periods varying from 30 min to 24 hours. As opposed to the suggestive data of Table 16, no significant reactivation was observed. It should be pointed out that the specific activity, calculated in terms of umoles of carbon dioxide produced per mg protein per hour, obtained after dialysis against a buffer containing 0.2 M KCl, was approximately 10 times the specific activity calculated from the data in Table 8.

The effect of short term dialysis against dilute Tris-HCl buffer (pH 7.5) is shown in Figure 9. Samples of dialyzed enzyme were assayed as follows: (1) enzyme was held at 4 C during the course of the experiment, and assayed in the complete reaction mixture (as in Fig. 7), (2) enzyme assayed with pyridoxal phosphate at the routine level and 0.143 M KCl, (3) enzyme assayed with no added KCl, (4) enzyme assayed with KCl, but no added pyridoxal phosphate, and (5) enzyme assayed with neither pyridoxal phosphate or KCl. The data from the experiment indicated the rapid loss of pyridoxal phosphate

Figure 9. Effect of short term dialysis against dilute Tris-HCl buffer. Enzyme assay was carried out as shown in Figure 7, except that 0.4 µmole pyridoxal phosphate was used and except where altered by experimental design. Specific activity was defined as umoles CO_2 per mg protein per min. Symbols: no treatment (△), complete reaction mixture plus 0.143 M KCl (final concentration) (○), complete reaction mixture (●), minus pyridoxal phosphate but containing 0.143 M KCl (□), and minus pyridoxal phosphate (■).


Specific Activity (x10³)
0
5
10
15
20
25
Time (min)
0
30
60
90
120
150
180

from a crude 2-methylalanine decarboxylase preparation. With enzyme dialyzed for three hours slight restoration of activity was achieved by the addition of KCl, while the addition of pyridoxal phosphate restored 63 per cent of the activity observed in the undialyzed enzyme. Almost complete reactivation occurred when pyridoxal phosphate and KCl were added together. No reactivation occurred with enzyme dialyzed for periods longer than 4 to 5 hours, using both pyridoxal phosphate and KCl. Undialyzed enzyme could not be activated with KCl.

Table 17 indicates the degree to which various salts were effective in the restoration of enzyme activity after dialysis for a short period of time against 10^{-4}M phosphate buffer (pH 7.5). It is interesting that lithium and sodium ions were inhibitory to the system while potassium ions stimulated the enzyme and, in fact, restored 95 per cent of the specific activity (activity being measured as described previously but expressed on a per minute basis). Ammonium chloride was neither inhibitory nor stimulatory while cesium and tetramethylammonium chloride were slightly inhibitory. The rubidium salt was not available for this study.

That potassium ions may be important for reasons other than their contribution (together with the chloride ion) to the ionic strength of the medium is evidenced by data in Table 17 and by the fact that dialysis against 0.2 M tetramethylammonium chloride, buffered with 10^{-3} M Tris-HCl (pH 7.5), resulted in the loss of 70 per cent of enzyme

Table 17

Effect of monovalent cations on enzyme dialyzed for 120 minutes against Tris-HCl buffer

Salt added[a]	Activity[b]
No salt	4.07
Lithium chloride	0.0
Sodium chloride	0.0
Potassium chloride	5.37
Cesium chloride	3.00
Ammonium chloride	3.98
Tetramethylammonium chloride	2.87

[a]Added to a final concentration of 0.14 M.

[b]Expressed in μmoles CO_2 per 30 min, assayed as shown in Figure 9.

activity after nine hours. A similar experiment carried out against 0.2 M KCl indicated that only 10 per cent of the initial activity (as specific activity) was lost after 35 hours of dialysis.

Inhibition of 2-methylalanine decarboxylase by the sodium ion was found to be concentration dependent (Table 18). This inhibition, however, was easily reversed by the potassium ion. It was observed that the relative inhibition of 86 per cent of the enzyme activity caused by the inclusion of 0.107 M (final concentration) sodium chloride could be completely reversed by the addition of 0.036 M (final concentration) potassium chloride.

Effect of heat on crude 2-methylalanine decarboxylase

If the potassium ion acted through a stabilization of 2-methylalanine decarboxylase, one might expect a change in thermostability of the enzyme with increasing potassium ion concentration. The heat inactivation profile of crude 2-methylalanine decarboxylase, carried out in phosphate buffer (pH 7.5), is shown in Figure 10. It is clear that 0.5 and 1.0 M KCl (final concentration) can protect the enzyme; in fact, a slight activation occurred at about 50 and 60 C, respectively. Complete loss of activity, both with and without KCl, appears to occur at 68 to 72 C under these conditions.

In another experiment (Fig. 11) a more precise change in thermostability was demonstrated. Crude 2-methylalanine decarboxylase was heated at 50 and 55 C (in two separate experiments) for increasing

Table 18

The inhibition of 2-methylalanine decarboxylase by the sodium ion and the reversal of inhibition by the potassium ion[a]

Molar concentration of salt added (final)	Sodium inhibition	Reversal of sodium inhibition (0.107 M) by potassium[c]
None	100.0[b]	14.0[d]
0.014	69.5	-----
0.036	51.0	106.0
0.072	41.5	101.0
0.107	16.8	102.0
0.143	3.2	101.0

[a]Enzyme assayed as shown in Figure 9.

[b]Expressed as per cent of enzyme activity at increasing concentrations of sodium chloride.

[c]All experimental flasks contained 0.107 M (final concentration) sodium chloride.

[d]Expressed as per cent of enzyme activity at increasing concentrations of potassium chloride.

Figure 10. Heat inactivation profile and effect of different levels of KCl on heat inactivation of crude 2-methylalanine decarboxylase. Enzyme assay was carried out as shown in Figure 9. Symbols: no added salt, heated for 15 min (●), no added salt, heated for 5 min (●), 0.2 M KCl, heated for 5 min (O), 0.5 M KCl, heated for 5 min (△), 1.0 M KCl, heated for 5 min (□).

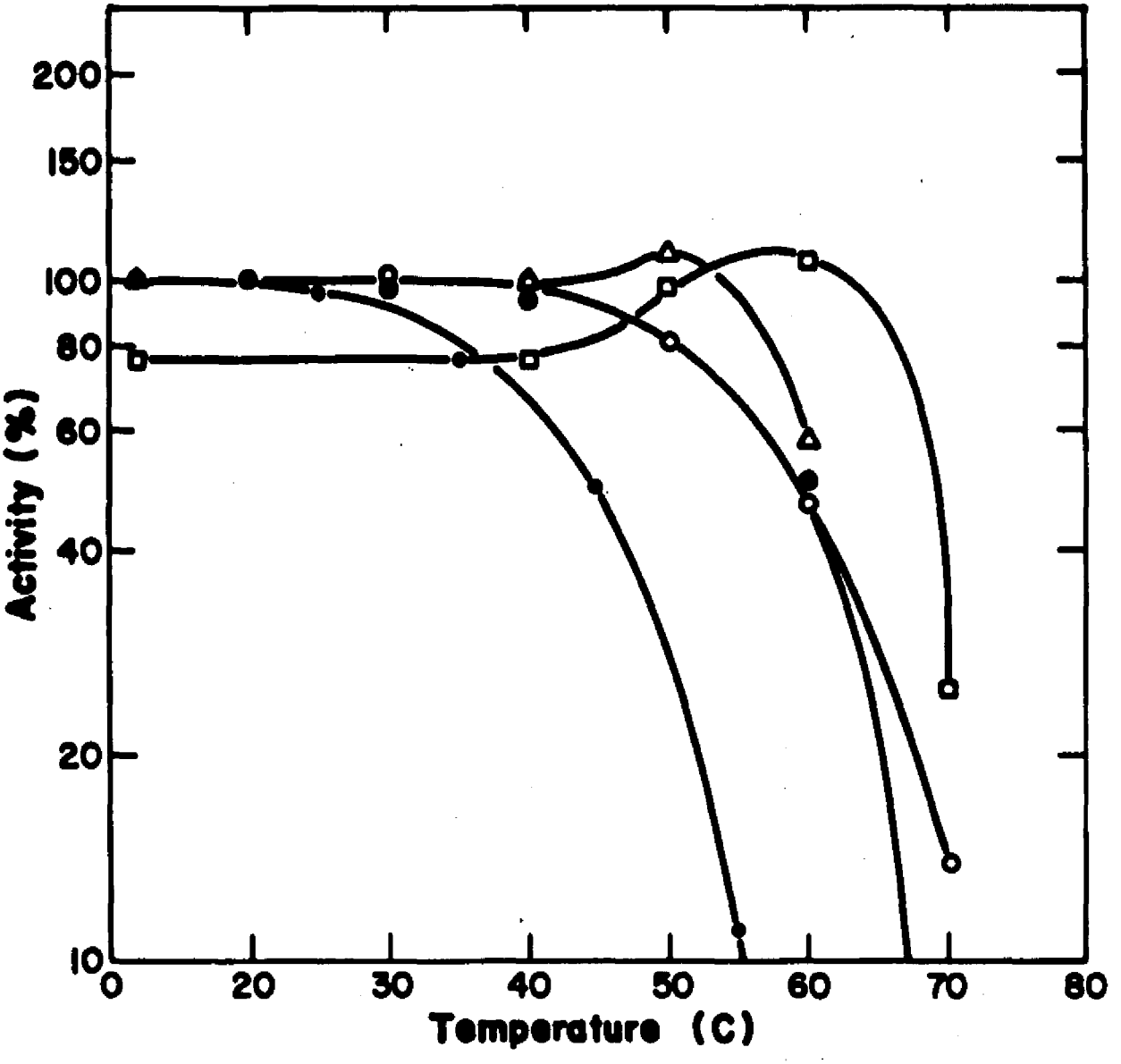
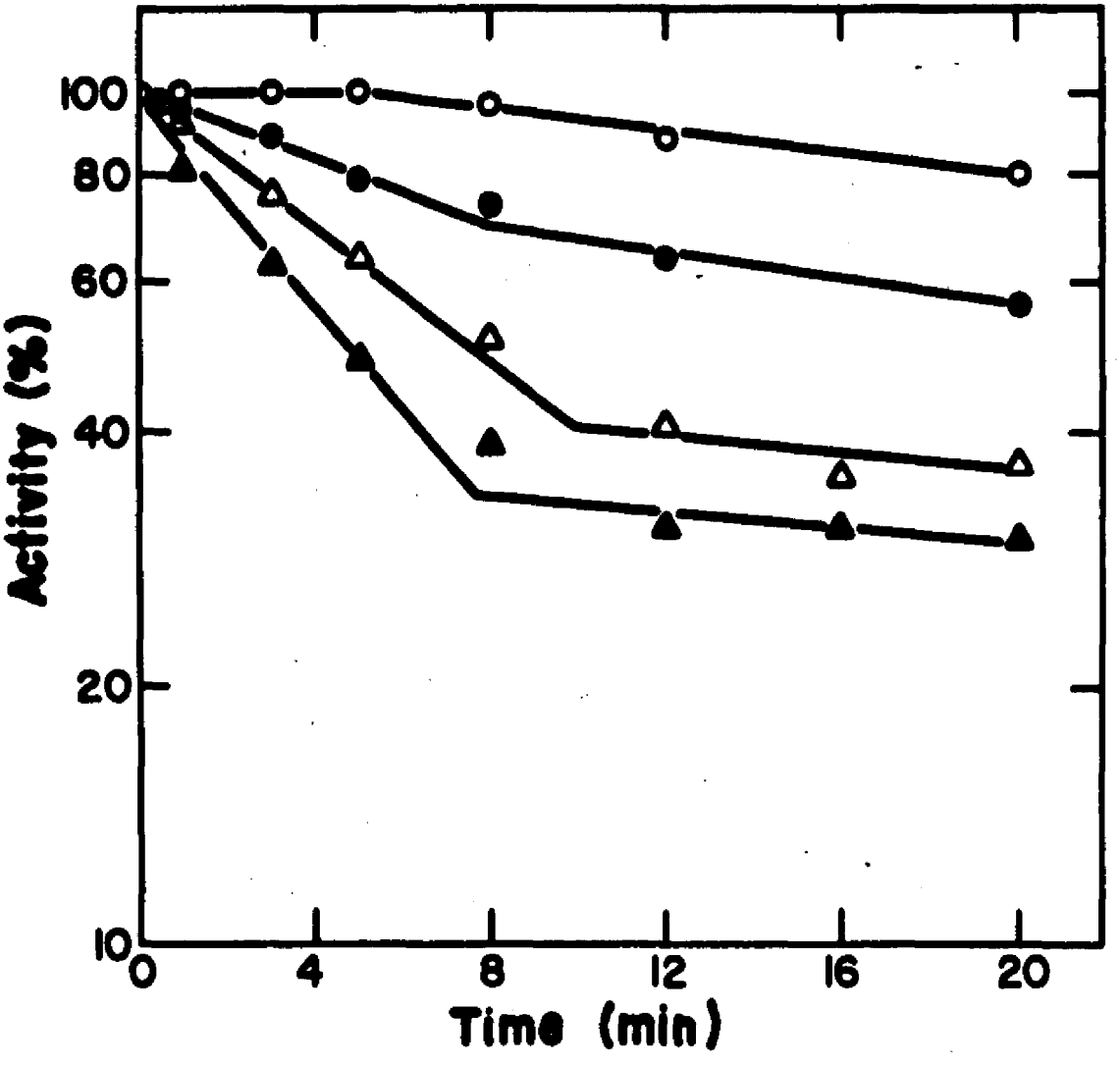

200
150
100
80
60
40
20
10
Activity (%)
0
20
30
40
50
60
70
80
Temperature (C)

Figure 11. Kinetics of heat inactivation of the 2-methylalanine decarboxylase activity. Enzyme assay was carried out as shown in Figure 9. Symbols: 50 C in the presence of 0.4 M KCl (O), 50 C with no added salt (●), 55 C in the presence of 0.5 M KCl (△), and 55 C with no added salt (▲).


100
80
60
40
20
10
Activity (%)
0
4
8
12
16
20
Time (min)

periods of time in the presence and absence of KCl. It is apparent that the slope of the curves increase in the absence of KCl and, further, that the inactivation curves are biphasic. Also it should be noted that the more thermostable portions of the curves are nearly parallel. These data demonstrated a stabilization of the enzyme by potassium and that heat inactivated either multiple components or sites on the enzyme.

The use of a 20 minute heat treatment at 50 C permitted a relative measure of the ability of different salts, substrates, pyridoxal phosphate, and reaction products to protect the enzyme from temperature inactivation (Table 19). The same pattern of inhibition by sodium and lithium ions (Table 17) may be seen in the activity of unheated enzyme. It is clear that the bromine and iodine anions are inhibitory to the system. It should be noted that even those salts that were inhibitory were able to protect against heat inactivation. Tetramethylammonium chloride is of particular interest since its effect may be attributed solely to ionic strength. It is significant that the coenzyme, pyridoxal phosphate, protected to an extent of 84.5 per cent and that pyridoxal phosphate, 2-methylalanine, and KCl in combination protected 93.2 per cent of the initial (unheated) activity during heating at 50 C for 20 minutes. Apparently the catalytic site is protected under these conditions. Practical use of this study was made in purifying the 2-methylalanine decarboxylating enzyme or enzymes.

Table 19

Activity remaining after heating cellular extract at 50 C for 20 minutes in the presence of various salts and reaction mixture constituents

Addition[a]	Activity[b] Unheated	Activity[b] Heated	Per cent activity remaining[c]
None	6.70	3.70	55.0
0.2 M KCl (1)	6.37	5.06	79.5
0.4 M KCl	6.67	5.31	79.5
0.4 M KBr	5.60	3.58	64.0
0.4 M KI	3.45	2.70	78.3
0.4 M NaCl	4.23	2.68	63.3
0.4 M LiCl	1.96	1.43	73.0
0.4 M NH_4Cl	5.50	2.77	50.5
0.4 M $(CH_3)_4NCl$	5.85	4.21	72.0
10^{-4} M PLP[d] (2)	6.34	5.36	84.5
10^{-3} M 2-MA[d] (3)	6.73	4.73	70.5
10^{-3} M Pyruvate	6.30	4.64	73.7
10^{-3} M Acetone	5.78	4.37	75.5
(1), (2), and (3)	6.65	6.20	93.2
None, dialyzed 4 hr	3.02	0.94	31.0
0.4 M KCl, dialyzed 4 hr	3.18	3.40	107.0

[a]Enzyme was added to the salt, coenzyme, substrate, or reaction product, mixed and held for 90 minutes at 4 C before heat treatment.

[b]Expressed as μmoles CO_2 per 0.5 ml enzyme per 30 min.

[c]Relative to unheated preparation.

[d]PLP is pyridoxal phosphate, 2-MA is 2-methylalanine.

Purification of 2-methylalanine decarboxylase

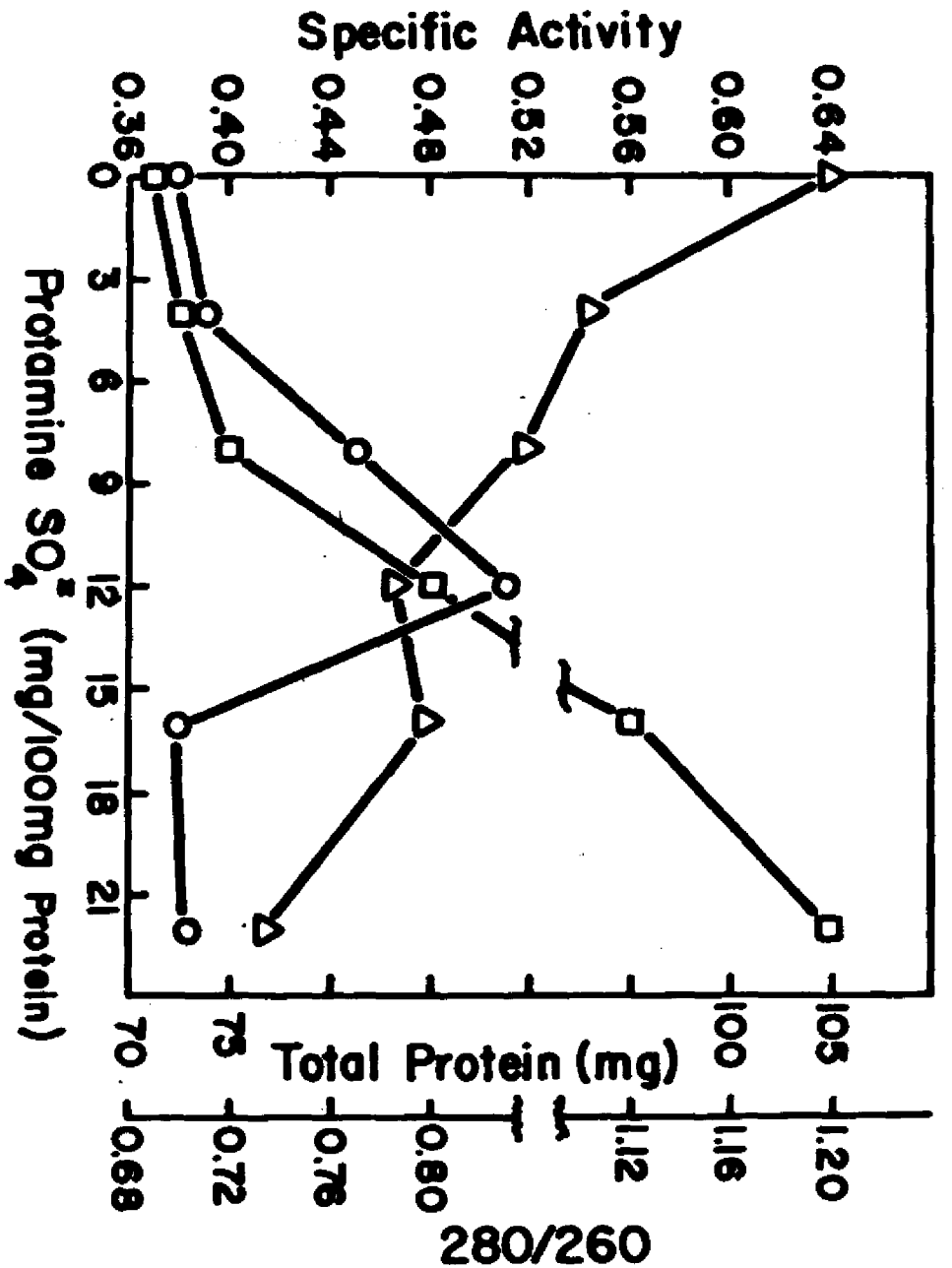
Numerous attempts to purify the pertinent enzyme or enzymes responsible for the decarboxylation of 2-methylalanine were made during the course of this study. These attempts were often unsuccessful but some success was achieved after characterizing the enzyme in the crude state. An example of this was the determination of the amount of protamine sulfate required to precipitate nucleic acids (Fig. 12). This experiment was carried out by adding dropwise increasing amounts of protamine sulfate (2 per cent in 0.1 M phosphate buffer, pH 7.0), to 5.0 ml of cellular extract. The protamine sulfate treated extracts were allowed to stand in the cold for one hour and then were centrifuged at 15,000 rpm in the Servall RC-2 centrifuge (rotor SS-34) for 30 minutes. The supernatant liquid was decanted and assayed for 2-methylalanine decarboxylase activity, protein content, and relative amount of nucleic acid (280/260 mμ absorbance). These results indicated that the addition of 12 mg of protamine sulfate per 100 mg of protein in the cellular extract resulted in reduction of the level of nucleic acids and a slight increase in the specific activity of the enzyme.

The protocol followed in a representative purification scheme was as follows:

(1) Preparation of the cellular extract (as described in the Materials and Methods section).

(2) Centrifugation in the Spinco Model L ultracentrifuge (rotor #50) for one hour at 30,000 rpm.

Figure 12. Determination of the level of protamine sulfate to use to remove nucleic acids from cellular extracts. 2-Methylalanine decarboxylase assayed as shown in Figure 9. Specific activity defined as μmoles CO_2 per mg protein per 60 min. Symbols: specific activity (○), total protein (△), and 280/260 (□).


Specific Activity
0.36
0.40
0.44
0.48
0.52
0.56
0.60
0.64
0
3
6
9
12
15
18
21
Protamine $SO_4^=$ (mg/100mg Protein)
70
75
100
105
Total Protein (mg)
0.68
0.72
0.76
0.80
1.12
1.16
1.20
280/260

(3) Treatment of the clear supernatant liquid from step (2) with 12 mg of protamine sulfate per 100 mg of protein, as described above, followed by centrifugation in the Servall RC-2 centrifuge (rotor SS-34) at 15,000 rpm (27,000 x g) for 20 minutes. Centrifugation in this manner was used routinely where required in the rest of this scheme.

(4) Addition of KCl and pyridoxal phosphate (0.5 and 10^{-4} M final concentration, respectively) to the supernatant liquid from step (3) followed by heat treatment at 50 C for five minutes. The sediment after centrifugation was extracted with a small volume of 0.05 M phosphate buffer (two times) and pooled with the supernatant solution.

(5) Selective precipitation of protein fractions with solid ammonium sulfate (enzyme grade, obtained from Nutritional Biochemicals Corp.) after the addition of 20 per cent (v/v) 1.0 M phosphate buffer (pH 7.5) and adjusting the protein content to 10 mg/ml. The enzyme was found in the 0.00 to 0.45 saturation fraction; it was dissolved in a minimal volume of 0.05 M phosphate buffer.

(6) Dialysis against 10^{-4} M phosphate buffer (pH 7.5) containing 0.2 M KCl until ammonia was no longer detected by Nesslers reagent, or the passage of the ammonium sulfate fraction through a Sephadex G-25 column equilibrated with the above buffer.

(7) A second treatment with protamine sulfate as described in step (3).

(8) A second precipitation with ammonium sulfate as described in step (4), followed by centrifugation and dialysis. The greatest amount of the enzyme was found in the 0.30 to 0.60 saturation fraction; however, some activity was in the 0.00 to 0.30 fraction.

A summary of pertinent data obtained during the course of purifying 2-methylalanine decarboxylase is shown in Table 20. An overall purification of 10-fold was achieved with 59 per cent of the enzyme activity recovered. These data suggested that the 2-methylalanine decarboxylating enzyme may be a single protein.

Density gradient and kinetic analysis of partially purified 2-methylalanine decarboxylase

The results of a linear 5-20 per cent sucrose density gradient (also containing 0.2 M KCl) centrifugation study are shown in Figure 13. A volume of 0.2 ml of partially purified enzyme (through the first ammonium sulfate fractionation), containing about 4.0 mg of protein, together with about 40 μg of crystalline catalase, was carefully layered on top of the gradient. A Spinco Model L-2 ultracentrifuge was employed at 35,000 rpm (rotor SW-39) for 16 hours. The temperature was held at 3 C. A total of 34.7 fractions, each containing 10 drops, were collected by puncturing the bottom of the lusteroid tube. The even-numbered

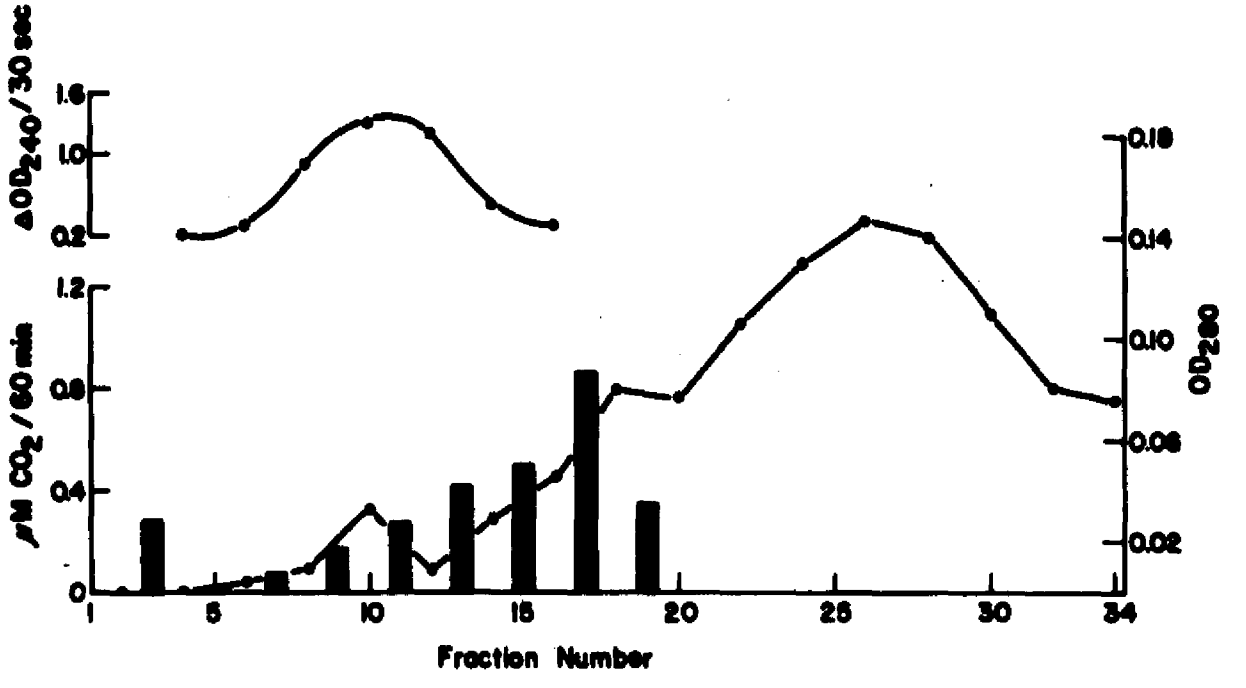
Table 20

Purification of 2-methylalanine decarboxylase

Fraction	Protein			Specific activity[a]	Total units	Recovery per cent	Fold purified
	Volume ml	mg/ml	Total mg				
1. Cellular extract	102.0	20.2	2040	0.022	44.8	100.0	----
2. High speed supernatant	92.5	18.4	1700	0.024	40.8	91.0	1.14
3. Treatment with protamine sulfate	99.0	15.6	1540	0.027	41.5	92.5	1.23
4. Heated at 50 C for 5 min[b]	93.0	10.4	970	0.038	37.4	83.5	1.68
5. Ammonium sulfate precipitation							
a. 0.0 - 0.45	14.1	24.0	338	0.072	24.3	----	3.29
b. 0.45 - 0.65	12.1	24.0	290	0.0	----	----	----
c. 0.65 - 0.90	13.4	14.0	188	0.005	0.9	----	----
6. Treatment of 5a. with protamine sulfate[c]	23.3	12.0	280	0.119	33.3	74.0	5.40
7. Ammonium sulfate precipitation							
a. 0.0 - 0.30	4.3	16.8	72	0.114	8.2	18.3	----
b. 0.30 - 0.60	7.2	24.0	173	0.215	26.6	59.4	9.80
c. 0.60 - 0.90	3.1	4.0	12	0.100	1.2	2.7	----

[a]Specific activity defined as μmoles CO_2/mg/min.
[b]Heated in the presence of 0.5 M KCl.
[c]Buffer extracted sediment from step 4 added back before treatment with protamine sulfate.

Figure 13. Sucrose (5-20%) density gradient analysis of partially purified 2-methylalanine decarboxylase. See Results section for experimental details. Enzyme assay was carried out as shown in Figure 9. Catalase was assayed for by a change in absorbance at 240 mμ. Protein profile was determined by absorbance at 280 mμ. Symbols: 2-methylalanine decarboxylase activity (bar diagram), catalase (●), and protein (O).


ΔOD240/30 sec
1.6
1.0
0.2
μM CO2 / 60 min
1.2
0.8
0.4
0
OD280
0.18
0.14
0.10
0.06
0.02
1
5
10
15
20
25
30
34
Fraction Number

fractions were assayed for catalase with H_2O_2 as substrate by measuring the change in absorbancy at 240 mμ in the Beckman Model DB spectrophotometer. The odd-numbered fractions were assayed for 2-methylalanine decarboxylase. The fact that the 2-methylalanine decarboxylating enzyme can be separated from the bulk of the protein applied to the gradient is clear from these data; moreover, it would appear that one enzyme is responsible for the decarboxylation dependent transamination of 2-methylalanine.

If one assigns a sedimentation constant of 11.3 Svedberg units to catalase and using the dimensions reported by Martin and Ames (1961) for the SW-39 rotor, the sedimentation constant of 2-methylalanine decarboxylase may be calculated to be 8.5. Assuming that the enzyme is spherical and has a partial specific volume of 0.725 cm^3/g and taking the molecular weight of catalase as 250,000, a molecular weight of 142,000 for 2-methylalanine decarboxylase is obtained.

Finally the data for the calculation of the Michaelis constants for the two substrates, 2-methylalanine and pyruvate, and the coenzyme, pyridoxal phosphate, are presented in Figures 14, 15, and 16. The enzyme preparation used for this study was 10-fold purified and had been dialyzed against a buffer containing 0.2 M KCl. The Michaelis constants were taken from double reciprocal plots (Lineweaver and Burk, 1934). The Michaelis constants for 2-methylalanine and pyruvate should be termed apparent Michaelis constants since they were obtained

Figure 14. Double reciprocal plot for the determination of the Michaelis constant of 2-methylalanine. The velocity was measured as the μmoles CO_2 produced during 15 minutes. The reaction mixture contained: 2-methylalanine (1.4 to 56 μmoles), 20 μmoles of sodium pyruvate, 0.5 μmoles of pyridoxal phosphate, 0.5 ml of 0.05 M Tris-HCl buffer, pH 7.8, and 0.3 ml of enzyme from step 7b. of Table 20 (the enzyme preparation had been dialyzed against 0.2 M KCl). Distilled water was added to make a final volume of 2.8 ml; 0.2 ml of 10% TCA was added to stop the reaction and liberage the CO_2.

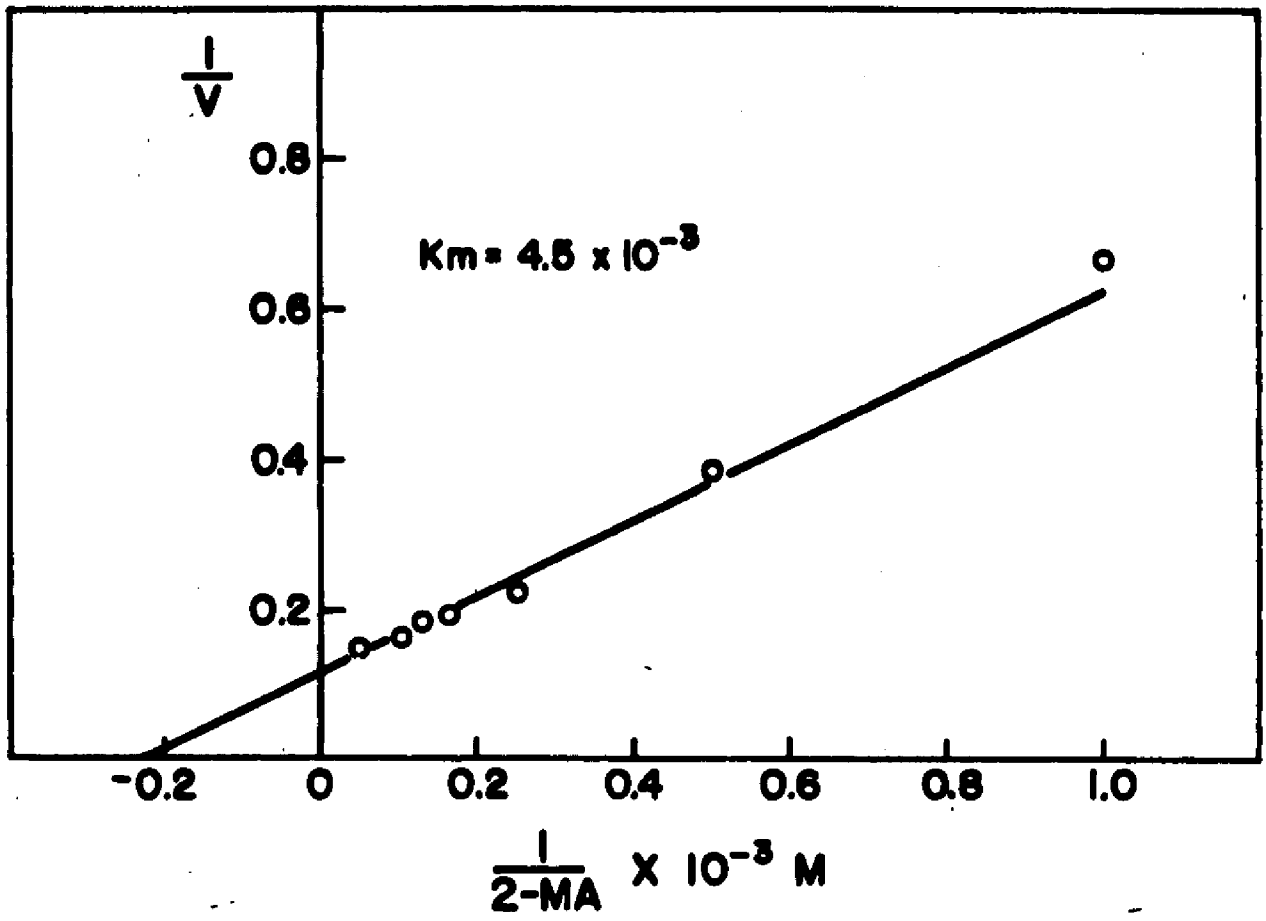

1/V
0.8
0.6
0.4
0.2
Km = 4.5 x 10⁻³
-0.2
0
0.2
0.4
0.6
0.8
1.0
1/2-MA X 10⁻³ M

Figure 15. Double reciprocal plot for the determination of the Michaelis constant of pyruvate. Experimental procedure was identical to that shown for Figure 14, except that pyruvate (sodium salt) was added to the reaction mixture over the concentration range of 1.4 to 22.4 μmoles. 2-Methylalanine was added at 30 μmoles.

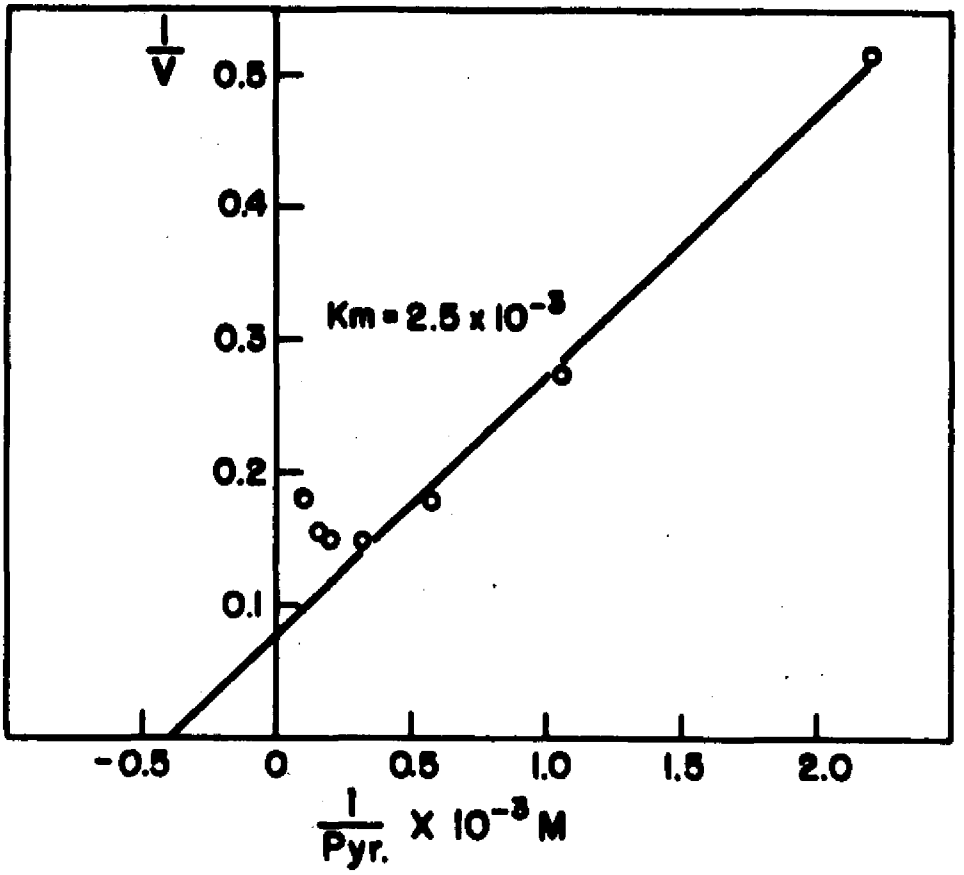
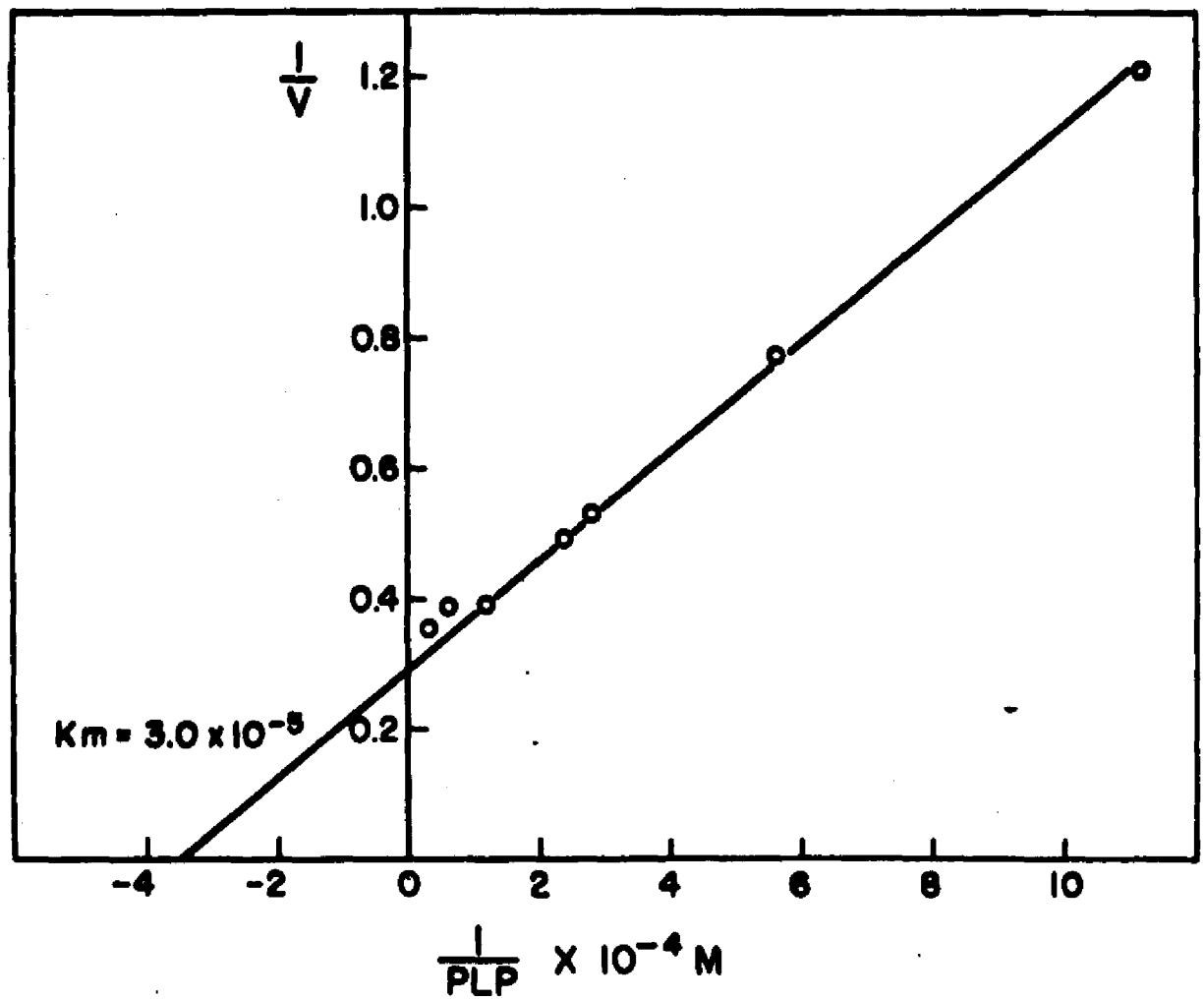

1/V
0.5
0.4
0.3
0.2
0.1
Km = 2.5 x 10⁻³
-0.5
0
0.5
1.0
1.5
2.0
1/Pyr. X 10⁻³ M

Figure 16. Double reciprocal plot for the determination of the Michaelis constant of pyridoxal phosphate. Experimental procedure was identical to that shown for Figure 14 except that the enzyme was purified beyond step 7b. of Table 20 by density gradient centrifugation. The enzyme was dialyzed 12 hours against buffered 0.2 M KCl. 2-Methylalanine and pyruvate were added at 30 μmoles and 20 μmoles, respectively.


1/V
1.2
1.0
0.8
0.6
0.4
0.2
Km = 3.0 x 10⁻⁵
-4
-2
0
2
4
6
8
10
1/PLP X 10⁻⁴ M

at saturating but not infinite levels of co-substrate. The Michaelis constant for pyridoxal phosphate was readily obtained due to the fact that pyridoxal phosphate could be easily removed by short term dialysis.

A Michaelis constant of 4.5×10^{-3} M was obtained for 2-methylalanine. This value was calculated from the data graphed in Figure 14; the co-substrate, pyruvate, and the coenzyme, pyridoxal phosphate, were at saturating levels. The upward swing of the curve shown for the experiment in which pyruvate was varied, at saturating levels of 2-methylalanine and coenzyme, may represent sodium inhibition (the highest level of sodium pyruvate added was 0.008 M) or substrate inhibition. A typical plot was obtained when pyridoxal phosphate was varied. The Michaelis constant for pyridoxal phosphate was calculated to be 3.0×10^{-5} M.

DISCUSSION

Enrichment culturing for the isolation of microorganisms specifically and exceptionally well-suited for a given environment has been employed in the field of bacteriology for over half a century. That 2-methyl amino acids were susceptible to metabolic attack was suggested by the early work of Ehrlich (1908), den Dooren de Jong (1926), and more recently by Christensen and Jones (1961). It was not surprising, therefore, that a bacterium capable of utilizing 2-methylalanine as a sole source of carbon could be isolated, even though this compound has been reported to be metabolically inert in mammalian tissues (Leighty and Corley, 1937; Noall et al., 1957).

The soil bacterium which was isolated during this study was classified as a member of the genus Pseudomonas. This classification was made after consideration of the possibility of assigning the isolate to one of the genera of the order Eubacteriales. At first, classification of the organism as a pseudomonad was questionable due to the lack of motility and pigmentation when cultured in a variety of media. However, the organisms' strong oxidative ability on carbohydrates and certain other biochemical characteristics led to the classification of the organism as a member of the genus Pseudomonas (Breed et al., 1957).

This classification may be subject to challenge because the classification of heterotrophic, Gram-negative, asporogenous, rod

or rod-like bacteria is presently somewhat confused. DeLey in a recent review (1964) states that classification of bacteria of the families Pseudomonadaceae and Achromobacteriaceae is not yet resolved. This author cites problems in characterization, "...only in a few cases can genera be recognized easily by one of a few striking properties..."; semantics, "...three standard manuals on bacterial classification... quite often provide different definitions for several genera and even different names..."; and classification, "...three standard manuals also differ considerably in their arrangements of the genera into tribes, families, and orders...."

On the basis of the determinative scheme proposed by Shewan, Hobbs, and Hodgkiss (1960) the 2-methylalanine-utilizing isolate could be placed in the genus Achromobacter. This was considered, as was the possibility of placing the bacterium in the genus Alcaligenes; however, the isolate appeared markedly dissimilar to the two respective type species (Breed et al., 1957). If the isolate were classified as an achromobacter, it most closely resembles Achromobacter parvulus. Alcaligenes metalcaligenes is that member of the genus Alcaligenes which is most similar to the 2-methylalanine isolate.

The mechanism by which the organism is able to grow at the expense of 2-methylalanine appears to involve a decarboxylation dependent transamination of 2-methylalanine. The products of this reaction are carbon dioxide, acetone, and the pyridoxamine form of

the involved enzyme. The pyridoxal-catalyzed cleavage of 2-methylalanine to carbon dioxide, acetone, and pyridoxamine (reaction 2 of Kalyankar and Snell, 1962) is considered to be a non-enzymatic precedent for the mechanism of 2-methylalanine catabolism suggested above.

The mechanism of the pyridoxal-catalyzed cleavage of amino acids to carbon dioxide and an amine, proposed by Metzler et al. (1954), ascribed no essential role to the αC-hydrogen atom of the amino acid. Mandeles et al. (1954) proved that the αC-hydrogen was retained during enzymatic decarboxylation in deuterium oxide and, therefore, was not essential for decarboxylation. The suggestion that the enzyme discussed in this dissertation operates by a mechanism very similar to or identical with a non-enzymatic model reaction is common in the field of pyridoxal phosphate enzymology. The hydroxymethyltransferase reported by Wilson and Snell (1962a,b), for example, represents the enzymatic equivalent of the non-enzymatic pyridoxal-catalyzed cleavage of 2-methylserine studied by Longenecker et al. in 1954. The demonstration of non-enzymatic and enzymatic systems amply support the credibility of enzymes active on methyl-substituted amino acids.

On the basis of the results reported in this dissertation one may visualize the mechanism as proceeding by Schiff's base formation between 2-methylalanine and the coenzyme, pyridoxal phosphate. The amino acid may then be decarboxylated, and the resulting substrate-coenzyme complex could be enzymatically directed into one of two

possible stabilized Schiff's base structures. One of these (structure III of Kalyankar and Snell, 1962) upon hydrolysis would form an amine, in this case isopropylamine, and the aldehyde form of the coenzyme; whereas the other Schiff's base (structure IV of Kalyankar and Snell, 1962) would yield a keto compound, acetone, and the amine form of the coenzyme after hydrolysis. Enzymatic control favoring the latter reaction could explain the results of the present study. The stimulatory effect of a keto acid, presumably required to regenerate the aldehyde form of the coenzyme by transamination, supports this view. Since no free isopropylyamine was detected in 2-methylalanine metabolizing systems, and since this compound was not oxidized by whole cells or cellular extracts, the enzymatic formation of isopropylamine and the regeneration of pyridoxal phosphate appear doubtful. It should be mentioned at this point that preliminary attempts to transaminate isopropylamine with cellular extracts was unsuccessful. The inhibitory effect of divalent cations is in accordance with the results of Kalyankar and Snell (1962) that divalent cations markedly inhibited the model decarboxylation dependent transamination system.

The decarboxylation dependent transamination of 2-methylalanine should proceed without oxygen uptake; therefore, oxygen uptake measured in whole cell experiments probably represents the oxidation of acetone. Reports in the literature indicate that in animal tissues acetone is actively metabolized. Using intact rats, Price and Rittenberg (1950)

demonstrated a cleavage of acetone to form a two carbon fragment, which entered the "acetyl" pool. Sakami (1950) has shown that the methyl groups of acetone may be used by rat tissues for the synthesis of the β-carbon of serine and the methyl groups of methionine and choline. Also using rat tissues, Rudney in 1954 showed that propanediol phosphate was an intermediate in acetone catabolism. In each of the above reports all three carbon atoms of acetone were found to be involved in carbohydrate synthesis.

The metabolism of (1,3-C^{14}) acetone by plant tissues was examined by Cossins in 1963. It was found that the labeled methyl groups were rapidly incorporated into carbon dioxide, organic acids, amino acids, and lipids. The presence of labeled acetate and formate in the organic acids extracted from acetone metabolizing plant tissues might indicate a splitting of acetone into one and two carbon fragments similar to that reported by Price and Rittenberg (1950).

The report of Levine and Krampitz (1952) that a soil diphtheroid was capable of oxidizing acetone is also significant. They found that the ability of the bacterium to oxidize acetone was adaptive, requiring growth of the organism in its presence. They mentioned, in fact, that cells obtained from cultures containing more than 0.01 per cent of yeast extract were incapable of oxidizing acetone. This phenomenon was observed in the case of 2-methylalanine oxidation (Table 4). Levine and Krampitz (1952) also showed, using adaptive enzyme and

isotopic experiments, that acetol and acetaldehyde but not propanediol were intermediates in the oxidation of acetone.

The manner in which the experimental organism catabolizes acetone is not known; however, the fact that the 2-methylalanine decarboxylase, the acetone oxidizing enzyme or enzymes, or both, represent adaptive enzymes is clear (Tables 4 and 5). It was observed that the enzyme system for the oxidation of acetone was rendered inactive upon the preparation of cellular extracts, and also that the ability of whole cells to oxidize acetone after being held for a period of weeks at -23 C decreased faster than their ability to decarboxylate 2-methylalanine.

The fact that 2-methylalanine decarboxylase could be purified 10-fold and sedimented in a 5 to 20 per cent sucrose gradient during centrifugation as a single active species suggests that the decarboxylase is a single enzyme. This is significant since both a decarboxylation and a transamination are catalyzed by the enzyme. In this connection the multifunctional characteristics of certain pyridoxal phosphate enzymes is of interest. Newton and Snell (1964) have described the multiple functions of a crystalline tryptophanase, prepared from E. coli, to include the α-β eliminations of water from L-serine, L-cysteine, and S-methyl-L-cysteine. Novogradsky, Nishimura, and Meister (1963) reported that a highly purified aspartic acid β-decarboxylase obtained from Alcaligenes faecalis acted both as a general amino acid transaminase and an aspartic acid

decarboxylase. In a subsequent publication Novogradsky and Meister (1964) determined that the same active site participated in both reactions, one of which (transamination) was capable of destroying or regenerating the coenzyme necessary for the other. The ratio of the rate of decarboxylation to transamination (with α-ketoglutarate) at pH 5.0 was 500; this value varied with pH. The authors suggest that this phenomenon may serve as a control on decarboxylase activity.

A curious aspect of pyridoxal phosphate catalysis is the fact that purified enzymes, unrelated in function to transaminases, can in some cases act as transaminases when supplied with pyridoxal phosphate. Waksman and Roberts (1963) have recently shown that diaphorase, beef heart lactic dehydrogenase, and yeast alcohol dehydrogenase, transaminate aspartic and glutamic acid at about five per cent the rate of purified aspartic-α-ketoglutaric transaminase. Phosphorylase A transaminated at 20 per cent of the rate of the transaminase described above.

That 2-methylalanine decarboxylase may be separated from the coenzyme, pyridoxal phosphate, so easily (Fig. 9) is important. This indicates that the pyridoxal phosphate is probably not bound to the enzyme by a Schiff's base through the terminal amino group of lysine. Such an attachment to the protein, as has been shown for other transaminases (Hughes, Jenkins, and Fischer, 1962), would bind the pyridoxal phosphate in a non-dialyzable form. Spectral examination

of a highly purified preparation of the enzyme for the characteristic absorbancy of the lysine-pyridoxal phosphate Schiff's base at 415 mμ (Dempsey and Christensen, 1962) would probably be negative.

It would appear from the data graphed in Figure 9 that KCl plays a role in the conformation of the active site. It is clear that 25 per cent more enzyme activity is attained when enzyme dialyzed for three hours is assayed in the presence of both KCl and pyridoxal phosphate, as opposed to the same enzyme assayed only in the presence of pyridoxal phosphate. Specificity for the potassium ion is apparent from the data shown in Table 17 and the fact that dialysis against 0.2 M tetramethylammonium chloride did not protect the enzyme. The manner by which the potassium ion acts is not known. An explanation on the basis of ionic strength, however, is felt to be incomplete.

Studies on the enzyme tryptophanase by Happold and Struyvenberg (1954) yielded results similar to those presented in this dissertation. These authors showed that apotryptophanase was activated by potassium, ammonium, and rubidium ions and pyridoxal phosphate, but was inhibited by sodium and lithium ions. Also the dissociation of pyridoxal phosphate from the enzyme occurred rapidly when the dialysis buffer was distilled water or 0.25 M sodium chloride, but only slowly when dialyzed against 0.25 M potassium chloride. Restoration of potassium ion and pyridoxal phosphate could lead to the reactivation of the enzyme dialyzed against dilute buffer only if added back during the first three hours of dialysis. 2-Methylalanine decarboxylase

displayed similar dialysis characteristics. Reactivation of 2-methylalanine decarboxylase which had been inactivated by dialysis or by passage through a Sephadex column equilibrated with dilute buffer could not be accomplished to any significant degree. Although Green (1964) found that threonine dehydrogenase could be reactivated (the enzyme had been inactivated by dialysis) by being held in the presence of KCl for long periods of time (12 to 24 hours), this procedure was not successful for 2-methylalanine decarboxylase inactivated by dialysis. The antagonism shown by sodium to this system has been reported for a number of other enzymes stimulated by the potassium ion (Dixon and Webb, 1964).

The change in thermostability of 2-methylalanine decarboxylase in the presence and absence of added KCl (Figs. 10 and 11) is consistent with the ability of potassium to stabilize and/or reactivate dialyzed enzyme. The heat inactivation profile and heat stimulation of 2-methylalanine decarboxylase in the presence and absence of KCl (Fig. 10) is similar to data reported by Morino and Wada (1963). These workers found that a glutamic-oxaloacetic transaminase prepared from beef liver mitochondria was stimulated 30 per cent when heated to 60 C in the presence of maleate, α-ketoglutarate, and pyridoxal phosphate.

The kinetics of heat inactivation (Fig. 11) show a marked change in thermostability in the presence of KCl at the two temperatures

investigated. The fact that these curves are biphasic indicates that two enzymes, enzyme subunits, or multiple sites on the enzyme were being affected. A similar plot presented by Schwartz and Bonner (1963) was used as partial proof that a tryptophan synthetase isolated from Bacillus subtilis was a two-component enzyme. The proposition that the enzyme 2-methylalanine decarboxylase is a single enzyme has been suggested on the basis of its purification and density gradient centrifugation studies. Moreover, if two enzymes were required to carry out the decarboxylation and transmination of 2-methylalanine, the coenzyme, in the amine form, would have to pass from the surface of the decarboxylase to the transaminase in order to return to the formyl form, and then pass back again to the decarboxylase. Such a shuttle mechanism does not appear probable to this author. Whether the 2-methylalanine decarboxylase enzyme is composed of subunits or not can not be proven with the present data; however, one might argue for the possibility that two sites on the surface of a single enzyme are responsible for the biphasic curves. Since pyridoxal phosphate was found to be bound so loosely to the enzyme and was apparently aided to some degree by the potassium ion in maintaining its functional orientation on the surface of the enzyme, it might be reasoned that the loose attachment of pyridoxal phosphate was designed to permit the coenzyme either to leave one site and pass to an adjacent one or to flip-flop through 180 degrees to another site. Speculation such as this is made credible by the complex nature of the reaction catalyzed by this enzyme.

2-Methylalanine decarboxylase is not specific with respect to the methyl-substituted amino acids which can serve as substrates. 2-Methylalanine and isovaline, amino acids which possess a hydrocarbon side chain, are the best substrates, while methyl amino acids carrying a thio-ether or hydroxymethyl side chain do not serve as well. The characteristic of the enzyme to accept amino acids with a hydrocarbon side chain is reflected in the keto acids used as co-substrates. It was observed that pyruvate, α-ketobutyrate, and α-ketovalerate would accept an amino group in the transamination half of the reaction, while oxaloacetate and α-ketoglutarate would not. The ability of whole cells to oxidize both isomers of isovaline is also significant in this regard. Apparently either a methyl or an ethyl group can fit the confirmation of the enzyme.

The sedimentation constant of 2-methylalanine decarboxylase was found to be 8.5 Svedberg units by employing density gradient centrifugation in a 5 to 20 per cent sucrose linear gradient. Using the method of Martin and Ames (1961) the molecular weight was calculated to be 142,000. The possibility that this molecular weight may be in error 10,000 to 20,000 due to the assumptions used in the calculation must be kept in mind. An additional method of gaining information on the molecular weight of the enzyme would be by analysis on a Sephadex column calibrated according to the methods of Whitaker (1963) or Andrews (1964).

Further purification of the enzyme might be possible by taking advantage of the size of the protein. Either the use of Sephadex G-200, or a similar molecular sieve, in column chromatography or the use of preparative density gradient centrifugation might permit additional purification to be achieved. Chromatography on cellulytic ion exchange resins might also be of value.

Preliminary experiments have indicated that a more rapid assay of 2-methylalanine decarboxylase may be possible. The enzyme activity would be assayed spectrophotometrically. The assay would consist of coupling the decarboxylation and transamination of 2-methylalanine with the formation of aminobutyrate from α-ketobutyrate. The aminobutyrate could then be measured by L-amino acid oxidase (assuming all of the aminobutyrate would be of the L-configuration). The oxidation of aminobutyrate could be followed spectrally at 600 mμ by the reduction of 2,6-dichlorophenolindophenol (Norton, Bulmer, and Sokatch, 1963). Such an assay would be of value since it would permit rate studies to be performed (the manometric assay being dependent upon the addition of TCA to the reaction mixture to liberate carbon dioxide) and since it would be an easy assay for monitoring the effluent from chromatographic columns. A rapid spectrophotometric assay would also be of value in performing kinetic studies.

This dissertation has reported on the demonstration of an enzyme capable of catabolizing the methyl-substituted amino acid 2-methylalanine.

More significant is the reaction this enzyme is considered to catalyze. The decarboxylation dependent transamination of 2-methylalanine is, as far as the author is aware, a unique biochemical reaction. It is important to note that this enzymatic reaction appears to follow a non-enzymatic model system. The systematic name 2-methylalanine: pyruvate carboxy-lyase (transaminating), 4.1.1.-, may be assigned to this enzyme on the basis of the rules of enzyme classification established by the International Union of Biochemistry (Dixon and Webb, 1964).

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VITA

Halvor Gunerius Aaslestad, the son of Captain and Mrs. Knut Aaslestad, was born on September 6, 1937 in Birmingham, Alabama. He was graduated from Francis T. Nicholls Public High School, New Orleans, Louisiana in 1955. He received the Bachelor of Science degree in Bacteriology in January, 1960 from the Louisiana State University, Baton Rouge, Louisiana. In July, 1960 he married Barbara Nell Wohn of Franklin, Louisiana. They have two daughters, Katherine and Karen.

The Pennsylvania State University, University Park, Pennsylvania awarded him the Master of Science degree in Bacteriology in December, 1961. From September, 1961 to May, 1962 he was employed by Difco Laboratories, Detroit, Michigan. During this period he traveled in the central Gulf States area as a manufacturer's technical representative.

In June, 1962 he reentered graduate school at the Louisiana State University. In February, 1964 he was awarded a predoctoral fellowship by the National Institute of General Medical Sciences, U.S.P.H.S. He is a member of the American Society of Microbiology and the Societies of Sigma Xi, and Phi Kappa Phi. He is a candidate for the Doctor of Philosophy in Bacteriology at the Louisiana State University, Baton Rouge, Louisiana.

EXAMINATION AND THESIS REPORT

Candidate: Aaslestad, Halvor Gunerius

Major Field: Bacteriology

Title of Thesis: The Bacterial Catabolism of 2-Methylalanine; Characteristics of the Enzyme 2-Methylalanine Decarboxylase

Approved:

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

Date of Examination:

May 12, 1965